ROLE OF SECOND GENERATION PHOSPHODIESTERASE INHIBITORS ON MAMMALIAN SPERM MOTILITY

by

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the University of Abertay for the degree of

Doctor of Philosophy

I certify that this is a true and accurate version of the thesis approved by the examiners, and that all relevant ordinance regulations have been fulfilled

Signed Principal Supervisor: ……. Date: …….……..

Declaration

I, Oladipo Anuoluwa Madamidola, hereby declare that this thesis is my own original work and has not been submitted elsewhere in fulfilment of the requirement of any other award. Where information has been derived from other sources, I can confirm that this has been indicated in the thesis.

Signed …… Date: ……..

SCHOOL OF SCIENCE, ENGINEERING AND TECHNOLOGY

DIVISION OF FOOD AND LIFE SCIENCES

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Doctor of Philosophy - THESIS

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ABSTRACT

Over three decades ago, W.H.O. declared infertility as a public health issue; due to its impact on millions of people worldwide. While cases of infertility could be multifactorial (affecting both male and female), 50% of cases are due to male factor infertility and this is mostly characterised by reduced sperm motility (asthenozoospermia). Assisted Reproduction Technology (ART) is the only treatment option available for this condition. Over 20 years ago, non-selective phosphodiesterase inhibitors (PDEi), such as pentoxifylline, were shown to enhance motility of human spermatozoa; however, contradictory results and stimulation of premature acrosome reaction has precluded their clinical use. Advancement in our knowledge have now made it clear that human sperm express several different PDEs and these are compartmentalised at different regions of the cells. By using type-specific phosphodiesterase inhibitors, differential modulation of sperm motility can be achieved without affecting other sperm function such as acrosome reaction. Additionally, by enhancing sperm function through PDE inhibition, there is a possibility of increasing IVF rates. The objective of this thesis is to: (1) examine the effect of phosphodiesterase inhibitors on spermatozoa in order to identify compounds that have clinically relevant enhancement of human sperm motility; (2) identify the signalling pathway(s) involved in the motility enhancing effects of identified compounds by targeting the modulator and mediator of cyclic nucleotides; (3) develop an animal IVF model to assess effects of Ibudilast on fertilization; and (4) optimise high performance liquid chromatography (HPLC) techniques for routine detection of cyclic nucleotides in sperm cells. A two phase drug screening approach was used to systematically and comprehensively screen series of compounds in order to identify those that have clinically relevant enhancement of human sperm motility. In phase 1, 6 compounds (out of 43 compounds) were found to have strong effects on poor motility samples, with magnitude of response ≥60% increase in percentage total motility. Additionally, these compounds significantly enhanced sperm penetration into cervical mucus substitute ($p \leq 0.05$), and they did not affect sperm acrosomal integrity nor cause externalisation of phosphatidylserine ($p=0.6$ respectively). 63% of IVF samples treated with compounds #26, #37 and #38 had significant increase in percentage total motility. For ICSI samples, compounds #26, #37 and #38 were the most effective. In respect to total motility, 88%, 81% and 79% of samples treated with these compounds showed significant increases in total motility, and 94%, 93% and 81% of samples showed significant increases in percentage of progressive cells,

respectively. Analysis of the signalling pathways, using PKA, sGC and PKG inhibitors, showed that chosen PDE inhibitors were working predominantly through PKA signalling pathways. Additionally, this study revealed that this pathway is needed for the maintenance of basal progressive motility and hyperactivation in human sperm. Animal IVF studies showed that addition of Ibudilast (compound #26) during sperm-oocyte incubation leads to higher IVF rates. Lastly, this study used an HPLC system to detect cAMP in boar sperm. This was done to explore if HPLC system can be used for high throughput detection of cyclic nucleotides in mammalian sperm.

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LIST OF ABBREVIATIONS

A23187	Calcium ionophore
AC	Adenylyl cyclase
AKAP	A Kinase anchoring protein
ALH	Amplitude of lateral head displacement
ANOVA	Analysis of variance
AR	Acrosome reaction
ART	Assisted reproductive techniques/technology
β	Beta
BSA	Bovine serum albumin
C	Catalytic subunit of protein kinase A
^{o}C	Degree Celsius
Ca^{2+}	Calcium ions
$[Ca^{2+}]i$	Intracellular calcium concentration
$CaCl_2$	Calcium chloride
CaM	Calmodulin
CaMK	CaM kinases
CCs	Cumulus cells

CM	Capacitating media
CO_2	Carbon dioxide
cAMP	Cyclic adenosine monophosphate
CASA	Computer assisted sperm analyser
cGMP	Cyclic guanosine monophosphate
COCs	Cumulus oocyte complexes
DIDS	4,4-diidothiocyanatostilbene-2,2-disulfonic acid
DMSO	Dimethyl sulfoxide
EGF	Epidermal growth factor
FSH	Follicle stimulating hormone
g	gravity
g	gram
GC	Granulosa cells
H89	N-[2-[[3-(4-Bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride
H^+	Hydrogen ions
HA	Hyperactivation
HCO_3^-	Bicarbonate ions
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HFEA	Human Fertilization and Embriology Authority
HPLC	High Performance Liquid Chromatography
Hz	Hertz
IBMX	3-isobutyl-1-methylxanthine
ICSI	Intracytoplasmic sperm injection
IUI	Intra Uterine Insemination
IVF	In vitro fertilization
IVF-m	IVF media
K^+	Potassium ions
KCl	Potassium chloride
KT5823	(9S,10R,12R)-2,3,9,10,11,12-Hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester
L or l	Litre
LH	Luteinizing hormone
LIN	Linearity
M	Molar
m	Milli (10^{-3})
mRNA	messenger RNA

µ	Micro (10^{-6})
min	Minute
$MgCl_2$	Magnesium chloride
NCM	Non-capacitating media
NO	Nitric oxide
OCM	Oocyte collection Media
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
OMM	Oocyte maturation media
PBS	Phosphate buffered saline
PDE(s)	Phosphodiesterase(s)
PDEi	Phosphodiesterase inhibitor
PDE1	Calmodulin-dependent phosphodiesterase
PDE2	cGMP- stimulated phosphodiesterase
PDE3	cGMP-inhibited phosphodiesterase
PDE4	cAMP-specific phosphodiesterase
PDE5	cGMP-specific phosphodiesterase
pFF	Porcine follicular fluid,
PKA	cAMP-dependent protein kinase A
PKC	Protein kinase C

PKG Protein kinase G

PSA-FITC Pisum sativum agglutin conjugated to fluorescein isothiocyanate

ROS Reactive oxygen species

sAC Soluble adenylyl cyclase

sGC Soluble guanylyl cyclase

shRNA small hairpin RNA

STR Straightness

VAP Average path velocity

VCL Curvilinear velocity

VSL Straight-line velocity

W.H.O World Health Organization

ZP Zona pellucida

Chapter 1.

General Introduction

1.1 Infertility and ART

Infertility remains a global epidemiological issue; and more couples are having trouble conceiving naturally. On average, the conception rate of "normal" fertile couples is 30% per month or approximately 85% per year and in circumstances where this rate reduces, couples are considered to be infertile. In the clinical sense, infertility is regarded as a disease of the reproductive system characterised by the failure to achieve a clinical pregnancy after twelve months of regular unprotected sexual intercourse (Cooper et al., 2010). Most cases of infertility can be categorised into: disorder of the female genital tract, disorders of ovulation, spontaneous abortion and seminal inadequacy. Although there are discrepancies in literature regarding the incidence of infertility, depending on what is considered reproductive success (e.g. pregnancy, on-going pregnancy and live birth) or definition of infertility (e.g. percentage of couples failing to conceive or time to pregnancy), male factor infertility is considered the single most common cause of infertility. Excluding regional variation, it is estimated that 3.7 million people (that is 1:7 couples) are affected in the UK (NICE 2013; HFEA 2013). Furthermore, male reproductive dysfunction is predominantly the main diagnosis in patients seeking treatment compared to other factors (Figure 1-1) and assisted reproductive technologies, such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are used to overcome some male factor infertility problems (HFEA 2013).

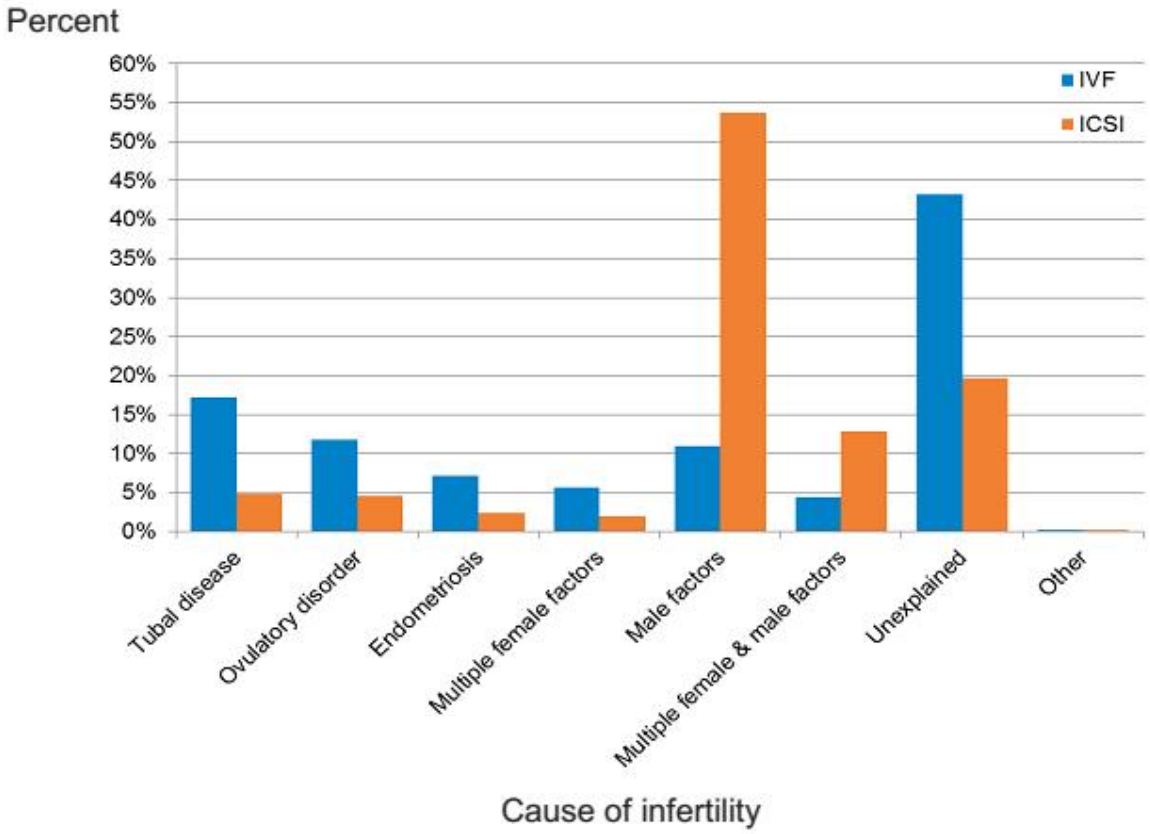


Figure 1-1: Graph indicating reasons for patients undergoing infertility treatment in 2013 in the UK (HFEA, 2013). Data shows male factor infertility as one of the most common cause of infertility.

With advances in assisted reproductive technology there has been an increase in success rates and there is less social and ethical stigma associated with this procedure. However, its economic and health consequences present issues for policy makers and health practitioners alike. In vivo, the female reproductive tract has been adapted to ensure the best sperm cells with the best genetic material are the ones with the best chance of fertilizing an egg. Although procedures such as ICSI and IVF effectively eliminate most male factor infertility issues (low sperm count or poor motility) by introducing sperm cells directly into or close to the proximity of an egg, they exclude the natural process of sperm selection in the female reproductive tract. Additionally, these procedures are now commercially driven and indiscriminate use without adequate diagnosis, even though there is no strong research evidence, has been associated with increased risk of developmental and congenital diseases

(Funke et al., 2010, Davies et al., 2012). Therefore, it's only with better understanding of different mechanisms, such as initiation of motility, capacitation, acrosome reaction, penetration and fusion with eggs, which regulates or is involved in sperm functions that male infertility could be appropriately diagnosed and treated.

Spermatozoa undergo complex developmental/ maturation processes as a result of interaction with the microenvironment in which they are produced and migrate. Immediately after ejaculation, mammalian sperm are unable to fertilize an oocyte. In vivo, the ability for fertilization is acquired in the female reproductive tract after further maturational process termed 'capacitation' (Austin, 1951, Chang, 1951). The process of sperm capacitation is still not completely understood; however, it involves plasma membrane reorganization resulting in increased membrane permeability, alkalization of sperm cytoplasm due to ion fluxes and activation of second messenger systems (A.P. Harrison and Gadella, 2005, Abou-haila and Tulsiani, 2009, Bou Khalil et al., 2006, Cross, 2004). After capacitation, spermatozoa must be able to bind the extracellular matrix of the egg (zona pellucida (ZP)) which leads to exocytosis of acrosomal content (induction of acrosome reaction) and finally, penetration and fusion with the vitelline membrane of an egg.

Although not fully characterised, many of the signal transduction pathways associated with sperm capacitation and acrosome reaction have been identified. It is widely acknowledged that extracellular calcium ions play a crucial role in the process of capacitation and acrosome reaction in mammalian spermatozoa (see section 1.3.2.1 and 1.5). In addition to this, there is evidence for increased intracellular cyclic nucleotides level (see section 1.3.3.1), activation of protein kinase A (PKA) (see section 1.3.3.3) and an increase in protein tyrosine phosphorylation (see section 1.3.3.3).

1.2 Spermatogenesis

Spermatogenesis is a complex, hormonally regulated, process of male germ cell proliferation and differentiation in the seminiferous tubules. This process is initiated at puberty and it is controlled by the pulsatile hypothalamic release of gonadotropin-releasing hormone (GnRH), follicle stimulating hormone (FSH) and luteinizing hormone (LH), which influence both the endocrine and paracrine stimulation of spermatogenesis. Within the testis, LH stimulates Leydig cells to synthesise testosterone, which with FSH, acts on Sertoli cells and other peritubular cells to release factors that fuel spermatogenesis(de Kretser et al., 1998).

The duration of spermatogenesis is estimated to be within 74 days in humans (Johnson, 1986) (41 days in boars (Franca et al., 2005)) and it involves three distinct phases, that is, spermatocytogenesis, spermatidogenesis and spermiogenesis. Spermatocytogenesis is a proliferative or a mitotic phase where non-differentiated spermatogonia give rise to two daughter cells (type A and B spermatogonia). While type A spermatogonia remain as stem cells, type B spermatogonia proliferate by mitosis to yield primary spermatocytes which go on to form secondary spermatocytes (meiosis I) (Phillips et al., 2010). The latter then proceed(s) to form round spermatids, in a process referred to as spermatidogenesis, during meiosis II. This cell division leads to conversion of diploid chromosomes in spermatocytes to haploid chromosomes in spermatids. Lastly, spermatids undergo dramatic transformation, during spermiogenesis, into streamlined spermatozoa adapted for fertilization (Figure 1-2). This transformation involves profound nuclear condensation and the movement of the nucleus to the periphery of the cell, formation of acrosomal cap which overlays the nucleus, and development of the flagellum responsible for sperm motility. The acrosome is derived from the Golgi apparatus and it contains secretory granules which are released during the fertilization process. It should be mentioned, however, that there are characteristic species-specific differences regarding the size, and shape of mammalian spermatozoa.

After transformations that occur through spermiogenesis, the functionally incompetent but anatomically "complete" spermatozoa are released into the long convoluted tubule known as the epididymis. The structure to function and biochemical characteristics of the epididymis have been highlighted in many reviews (Cooper, 2010). The human epididymis is segmented into eight sections (efferent ducts, anterior caput, posterior caput, anterior corpus, mid corpus, posterior corpus, anterior cauda, posterior cauda and ductus deferens) with distinct gene expression patterns and secretions into the intraluminal space by epididymal epithelial cells (Sullivan et al., 2011, Cornwall, 2009). Although it is widely accepted that it is during the passage through the epididymis that mammalian spermatozoa mature and acquire progressive motility and potential to fertilize the oocyte (Yeung et al., 1993, Yeung et al., 1997), others have argued for the redundancy of the epididymis on the basis of observation that spermatozoa from the proximal region of the tract has the potential to fertilize an oocyte both in vivo and in vitro (Bedford, 1994). Additionally, the relatively rapid transit that sperm undergo, in some cases, along the excurrent duct in men also argues against the notion of complex remodelling of sperm during its epididymal transit. Conversely, it has to be stated that the study questioning the relevance of epididymis was done in men that had undergone vasovasostomy, had a congenital lack of a vas deferens, or had an obstruction along the excurrent duct (Bedford, 1994). It has been postulated that the vas deference can possibly adapt to take on some of the function of the epididymis after re-anastomosis; overall, both proteomics and genomics analyses have given support to the fact that human epididymis does have a significant role in the functional maturation of spermatozoa (Cooper, 1993, Dacheux et al., 2006, Dacheux et al., 2009).

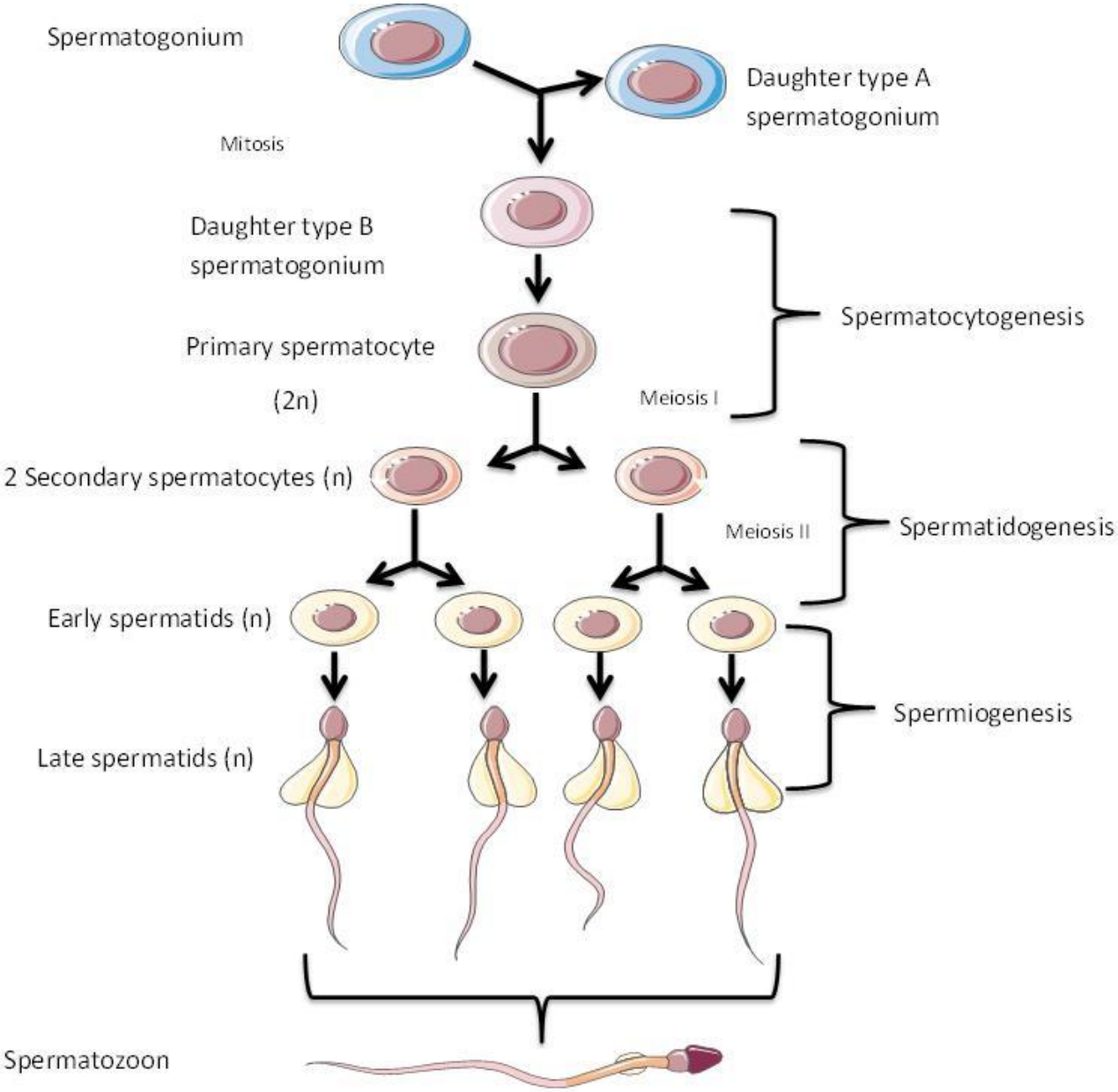


Figure 1-2: The process of spermatogenesis.

Animal studies have shown that a combination of low pH (<6.5) (Carr et al., 1985, Carr and Acott, 1984), low concentrations of sodium (Na^+) and high concentrations of potassium (K^+) (Cooper, 2010) or the presence of viscous mucoproteins in the epididymis (Mitra et al., 2010) (depending on species) are the primary mechanisms for maintaining the quiescence of mature spermatozoa prior to ejaculation. Upon ejaculation, spermatozoa become motile when they mix with the constituents of seminal plasma; this results in initiation of progressive motility. Sperm motility at this time is characterised by relatively low-amplitude, symmetrical tail bending when compared with the high amplitude, asymmetrical tail bending characteristic of hyperactivation (see section 1.4), which is observed close to the site of fertilization (Suarez and Pacey, 2006, Suarez, 2008). Although progressively motile, the cells are functionally in an inactive state without capacity to fertilize an oocyte.

1.3 Sperm Capacitation

Over six decade ago, two independent studies performed by Austin and Chang (Chang, 1951, Austin, 1951) showed that sperm cells need a period of incubation in the female reproductive tract to acquire the capacity to effectively fertilize an oocyte. The morphological and biochemical changes that occur between sperm deposition in the female reproductive tract and fertilization occurring is termed 'capacitation' (Austin, 1952). Although there is debate over which processes are encompassed by capacitation, some investigators stress the separation of capacitation and acrosome reaction (Chang, 1984). It is widely accepted that the capacitation process involves an increase in protein tyrosine phosphorylation, the acquisition of acrosomal responsiveness, and alterations in flagellar motility; which may be involved in the penetration of the zona pellucida. Despite the discovery of capacitation over 60 years ago, we have only recently begun to underpin, through *in vitro* studies, the molecular and biochemical modification that leads to capacitation. In many species, alterations in intracellular pH and calcium level are observed. Capacitation is also associated with

bicarbonate-sensitive increase in cAMP level, a nucleotide that is involved in the regulation of sperm motility, due to the presence of soluble adenylyl cyclase (sAC) (Figure 1-3).

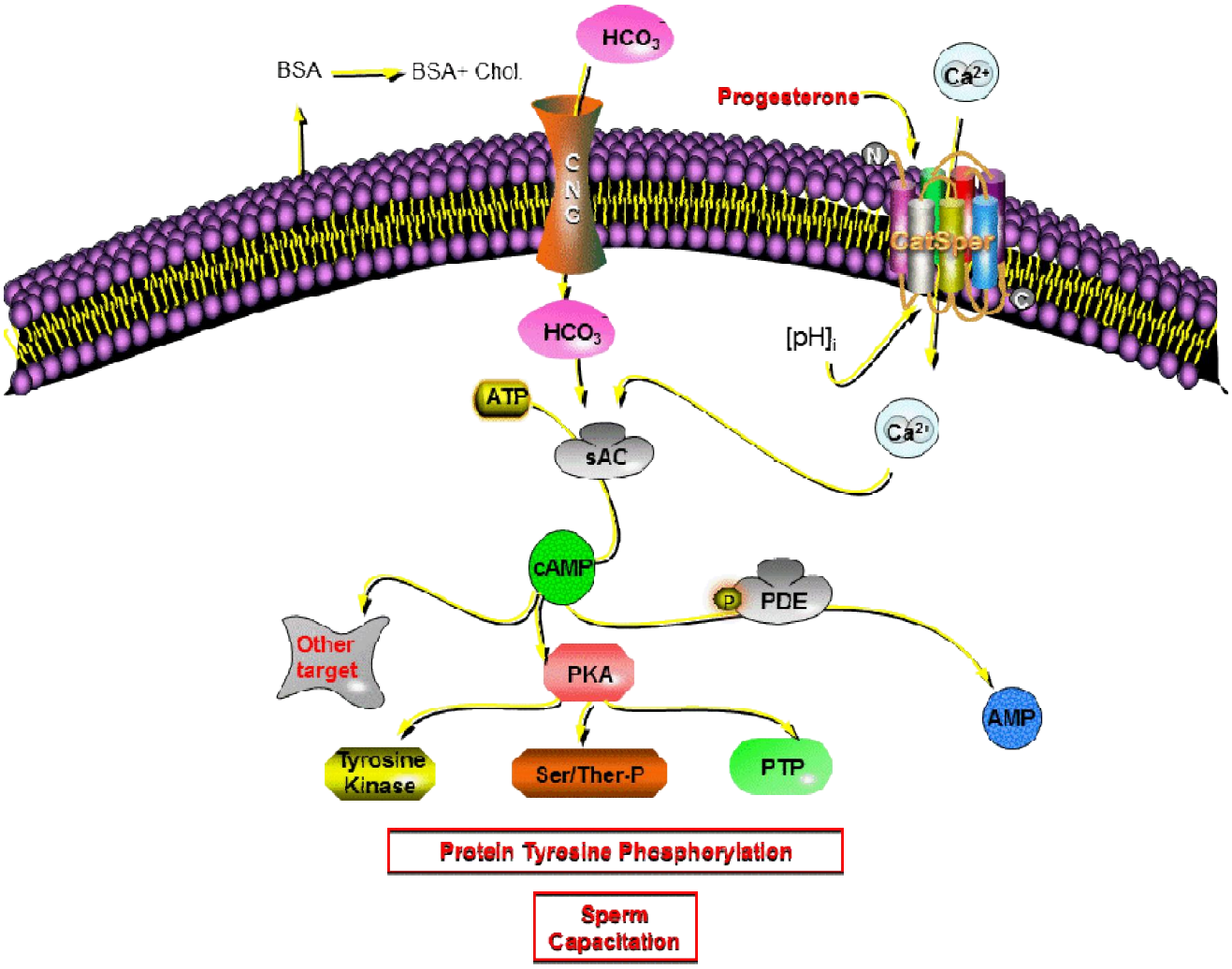


Figure 1-3: Intracellular signalling pathways involved in regulating sperm capacitation: Incubation of un-capacitated sperm under capacitating conditions gives rise to changes in the permeability of the sperm to Ca^{2+} and HCO_3^- via a change in membrane fluidity or membrane destabilization. These changes in membrane properties are thought to occur by the loss of cholesterol from the membrane; this loss may be accelerated by the presence of serum albumins, which can bind cholesterol. As a consequence of the increase in Ca^{2+} and HCO_3^- permeability, cAMP metabolism is altered. This could occur by the Ca^{2+} and/or HCO_3^- induced activation of adenylyl cyclase (AC), resulting in an increase in intracellular cAMP. The increase in cAMP then results in the activation of protein kinase A (PKA) which leads to phosphorylation of serine/threonine phosphorylation (Ser/Ther-P), activation of sperm tyrosine kinase(s) and/or inactivation of phosphoprotein phosphatases (PTP), the net result being an increase in protein tyrosine phosphorylation. As a consequence of an increase in protein tyrosine phosphorylation, events leading to capacitation and/or hyperactivation of motility are initiated.

1.3.1 Cholesterol Efflux and Initiation of Capacitation

Early events in the process of capacitation involve a major reorganization of the plasma membrane and the removal of cells from the decapacitating factors found in the seminal plasma. Seminal plasma contains decapacitating factors, such as semenogelin, that have regulatory influences on preventing sperm capacitation while maintaining the cells in a state of preparedness for capacitation (Cross, 1998, Mitra et al., 2010). For human spermatozoa, removal of unesterified cholesterol is a must before they can become responsive to an inducer of the acrosome reaction such as progesterone (Zarintash and Cross, 1996, Khorasani et al., 2000). It was suggested, although still debatable, that cholesterol regulates sperm function by preventing the exposure of specific antigens, such as the mannose-ligand, on the surface of plasma membrane (Martínez and Morros, 1996, de Lamirande et al., 1997b, Cross, 1998). The extraction of cholesterol has been proposed to cause mannose receptors to migrate into the phospholipid bilayer.

In a study examining the levels of cholesterol and phospholipids of fertile and unexplained infertile men, the cholesterol/phospholipid ratio in sperm of patients with unexplained infertility was found to be about twice that of fertile donors (Sugkraroek et al., 1991). Furthermore, sperm of normozoospermic men who failed to fertilize eggs in vitro were also found to be characterised by abnormally high cholesterol content or by a slow loss, or even an increase, in cholesterol during in vitro incubation (Benoff et al., 1993). The efflux of cholesterols from the plasma membrane changes membrane potentials, and increases membrane permeability and fluidity, this then allows an influx of ions; leading to the activation of secondary messengers such as cyclic adenosine monophosphate (cAMP) (Visconti et al., 1999a, Visconti et al., 1999b).

1.3.2 Ion Fluxes through Sperm Ion Channels

As mentioned above, efflux of cholesterol alters the fluidity and permeability of the plasma membrane and this leads to influx of ions (especially Ca^{2+} and HCO_3^- ions) into the intracellular domain of the cells. This influx of ions, mediated through the activation of ion channels, causes elevation of intracellular pH and initiation of signal transductions.

1.3.2.1 Calcium, CatSper Channel and Ca^{2+}Stores

Increase in intracellular Ca^{2+} ion is one of the main biochemical changes that occurs during sperm capacitation. There is accumulating evidence to suggest the prerequisite need of Ca^{2+} ion to modulate several events, including hyperactivation (Suarez, 2008), chemotaxis and acrosome reaction (Kirkman-Brown et al., 2002), in many species. As a result of new approaches and advances in the field, the mechanism by which Ca^{2+}-permeable channels participate in fertilization due to their distinctive localisation at different segments of the sperm cell are being elucidated (Darszon et al., 2005, Ren and Xia, 2010, Kirichok and Lishko, 2011, Kirichok et al., 2006). Since calcium is involved in many of the sperm cell processes; calcium homeostasis is crucial to sperm function in order to prevent premature initiation of signalling. The spatial distribution of these channels might have a physiological function of fine-tuning cell response to calcium influx; since different steps of fertilization have distinctive Ca^{2+} requirements (Marin-Biggiler et al., 2003, Bedu-Addo et al., 2008).

A sperm specific, pH sensitive ion channel has been localised in the principal piece region of human and mouse sperm tail (Quill et al., 2001, Ren et al., 2001, Navarro et al., 2008). The CatSper channels (CatSper1-4 Cationic channel of sperm), are putative transmembrane Ca^{2+} ion –channels with at least three auxiliary subunits: CatSperβ, CatSper δ, and CatSper γ (Chung et al., 2011, Liu et al., 2007, Wang et al., 2009). CatSper is synergistically activated by elevation of intracellular pH and extracellular progesterone (Lishko et al., 2011, Strünker et al., 2011, Nishigaki et al., 2014). Advances in mature sperm electrophysiology, through

whole-cell patch clamp technique, is elucidating the role of CatSper in influencing sperm cell behaviour (Kirichok and Lishko, 2011). CatSper has been identified as the only active calcium conductance present in mouse and human spermatozoa. The importance of this channel in relation to sperm function was confirmed by the generation of CatSper knockout mice. As a result of reduced ability to constitutively transport extracellular Ca^{2+} into the cells, sperm from these animals exhibit impaired motility and inability to hyperactivate, consequently, the animals were infertile because their sperm cells were unable to penetrate the zona pellucida of the oocyte. Also, studies have identified CatSper 1 and 2-mutations in human sperm and they revealed that the disruption to this gene is associated with male infertility (Hildebrand et al., 2010, Avenarius et al., 2009, Smith et al., 2013).

In a recent study by Tateno *et.al* the significance of Ca^{2+} was highlighted. In this study, by exposing mouse sperm to Ca^{2+} ionophore (20 μM), a compound that introduces a hydrophilic pore into the membrane, Tateno *et.al* suggested that short elevation of intracellular Ca^{2+} overcomes other necessary signalling pathways (Tateno et al., 2013). Although the results of this study are interesting, because A23187-treated spermatozoa recovered motility, showed hyperactivation, and were able to fertilize cumulus-intact eggs independent of cAMP-PKA pathway activation, the treatment conditions do not mimic *in vivo* conditions regarding calcium levels in the female reproductive tract and it betrays the time-dependence process of capacitation. It has to be made clear, however, that this observation has only been recorded in the mouse model.

In addition to extracellular Ca^{2+} influx via CatSper, evidence from human and animal studies showed that Ca^{2+} intracellular storage organelles are alternative sources of Ca^{2+} within the cells (Alasmari et al., 2013b, Costello et al., 2009, Bedu-Addo et al., 2008). Although the endoplasmic reticulum, which serves as Ca^{2+} store in somatic cells, is lost in sperm during spermatogenesis, the acrosome and the membranous organelles found at the sperm

neck/midpiece, serve as Ca^{2+} stores in sperm. Studies have shown that neck/midpiece Ca^{2+} store mobilisation is required to sustain sufficient Ca^{2+} elevation during CatSper channel mediated Ca^{2+} tail to head propagation (Olson et al., 2010). Mobilisation of stored Ca^{2+} in the neck/midpiece region of sperm is achieved through activation of two major intracellular receptor-operated Ca^{2+} channels: inositol triphosphate receptors (IP3Rs) and ryanodine receptors (RYRs) (Ho and Suarez, 2001, Park et al., 2011). A recent study by Alasmari et. al., using a combination of incubation conditions and pharmacological agent (activator and inhibitor of CatSper), showed that mobilisation of Ca^{2+} store at the sperm neck region induces a functionally different effects in human sperm and this is different to effects observed during CatSper activation. This study concluded that Ca^{2+} signalling as a result of Ca^{2+}store mobilisation stimulates flagellar activity and supports hyperactivation in human sperm (Alasmari et al., 2013b). The results from this study further highlight the complexity of signalling in human sperm and how distinct functions and behaviours are achieved through the activation and regulation of different pathways.

1.3.2.2 Bicarbonate Ions

Numerous studies have also shown that capacitation and many other sperm functions are bicarbonate (HCO_3^-) dependent (Lee and Storey, 1986, Boatman and Robbins, 1991, Shi and Roldan, 1995, Okamura et al., 1985). While a specific carrier protein responsible for the transport of bicarbonate into the sperm cell has not been unequivocally identified, the ability of 4,4-diidothiocyanatostilbene-2,2-disulfonic acid (DIDS), a well-known inhibitor of anion transporters, to inhibit the actions of HCO_3^- on various sperm functions suggests that sperm contain functional anion transporters (Okamura et al., 1988, Visconti et al., 1999c, Spira and Breitbart, 1992). In the female reproductive tract spermatozoa are exposed to higher bicarbonate levels, due to tubal epithelia secretions (Wang et al., 2003, Liu et al., 2012, Zhou et al., 2005), and this may influence the process of capacitation and hyperactivation. The

main functions attributed to the transmembrane movement of HCO_3^- are increase in intracellular pH, which might enhance the activity of transmembrane protein such as CatSper channel, and regulation of cAMP metabolism through stimulation of a unique type of adenylyl cyclase (sAC) (Visconti et al., 1999c).

1.3.3 Signal Transduction

1.3.3.1 cAMP and cGMP

Increase in the intracellular level of both HCO_3^- and Ca^{2+} leads to stimulation of adenylyl cyclase (AC) activity in sperm. Ten isoforms of AC have been identified in mammalian cells and adenylyl cyclase in sperm has been the subject of multiple studies. There are conflicting data about the nature of the mammalian sperm adenylyl cyclase. In fact, despite some studies describing the presence of the classical membrane adenylyl cyclase (mAC) (Leclerc and Kopf, 1995, Baxendale and Fraser, 2003, Harrison, 2003), recent findings demonstrate the prevalent expression of a distinct soluble adenylyl cyclase (sAC) (Chen et al., 2000, Litvin et al., 2003, Sinclair et al., 2000), localised to the flagellar region, which does not possess a transmembrane domain, is insensitive to forskolin and G protein regulation and is selectively activated by HCO_3^- (Hess et al., 2005). Stimulation of sAC generates a rapid and sustained increase in intracellular cyclic AMP levels which specifically activate protein kinases in different cellular compartments.

The role of cyclic adenosine 3', 5' monophosphate to indirectly influence mammalian sperm motility through activation of PKA is well established. In contrast, potential roles for cyclic GMP (cGMP) in mammalian sperm are less clearly defined. Cyclic GMP is produced from GTP as a result of a catalytic reaction by guanylyl cyclase (GC). GC is expressed both in soluble (sGC) and membrane (mGC) isoforms. The soluble isoform of guanylyl cyclase (sGC) contains a prosthetic heme group which has affinity for diffusible gases such as nitric

oxide (NO) and carbon monoxide (Wedel and Garbers, 2001). Consequently, NO can activate GC and thereby stimulates cGMP production. Indeed, human sperm has been found to contain nitric oxide synthase and endogenous production of nitric oxide has been suggested to be beneficial to human sperm motility through the activation of cyclic GMP/protein kinase G signalling pathway (Lewis et al., 1996, Miraglia et al., 2011). However, the beneficial effects of NO are only seen at a low concentration; with higher concentration having an adverse effect on spermatozoa as a result of NO acting as a free radical that cause direct oxidative damage to sperm cells (Rosselli et al., 1995, Zini et al., 1995).

In a study by Willipinski-Stapelfeldt *et. al,* the mean concentration of cGMP (0.1μM) was found to be 100-fold lower than that of cAMP in non-stimulated cells (10^7 cells). This result suggests that cAMP-dependent pathway might predominantly regulate motility and it further affirms the consideration that the cGMP-dependent pathway plays a comparatively minor role in normal mammalian sperm motility (Willipinski-Stapelfeldt et al., 2004). Additionally, reports on mammalian sperm cyclic nucleotide-gated ion channels that respond to cGMP (Ren et al., 2001, Weyand et al., 1994, Wiesner et al., 1998) and the identification of novel ion channels that are expressed specifically in sperm (Quill et al., 2001) raise the possibility that such proteins represent important target molecules for cGMP in human sperm.

1.3.3.2 Phosphodiesterase and Phosphodiesterase Inhibitors

Since cyclic nucleotides play a major role in mammalian sperm motility, regulating their levels are crucial (1) in order to prevent unwanted fluctuation in sperm motility, (2) to prevent premature hyperactivation, capacitation or acrosome reaction. The required equilibrium is achieved in mammalian spermatozoa by regulating the synthesis of cyclic nucleotides, through sAC and sGC, and degradation through phosphodiesterases. Cyclic nucleotide phosphodiesterases (PDE) are a family of enzyme that hydrolyses, cAMP and cGMP, to inactive 5′AMP and 5′GMP respectively. Mammalian PDEs are subdivided into 11

families, comprising 21 different gene products, with each member exhibiting a substantial difference in primary structure, tissue distribution and substrate specificity: some hydrolysing only cAMP (PDEs 4, 7 and 8), or cGMP (PDE 5, 6 and 9) and some hydrolysing both cAMP and cGMP (PDE 1, 3, 10 and 11) (Figure 1-4) (Francis et al., 2011). Therefore, the expression profile of PDEs within a given cell may determine the type of cyclic nucleotide hydrolysed in that cell or its subcellular regions. Besides, many of the PDE forms expressed in mammalian tissues are closely related to different physiological functions and pathological conditions. Hence, selective targeting of specific PDEs is a therapeutic means of treating some disease states (Gazzaruso et al., 2008, Vardi and Nini, 2007, Lipworth, 2005).

Currently, little is known about the presence, activity and subcellular localisation of specific PDE families in human spermatozoa. There is evidence for the presence of more than one isoform of PDEs in spermatozoa. A study by Richter *et. al*, reports the presence of mRNA transcripts of 6 types of PDE in ejaculated human spermatozoa (PDE 1A/B/C. 2, 3A/B, 4A/B/C, 5 and 8). However, since spermatozoa are considered to be transcriptionally and translationally quiescent, the role of these mRNAs in ejaculated spermatozoa is unclear (Richter et al., 1999). On the other hand, use of PDE inhibitors (PDEi) and the resultant stimulation of hyperactivation, capacitation and acrosome reaction has confirmed a role for PDEs in the control of sperm functions (Visconti et al., 1995b, Leclerc et al., 1996a, Fisch et al., 1998). PDE activity has been found in the head and flagellum of human spermatozoa respectively. Using selective inhibitors of PDE 1 and 4, MMPX and Rolipram, Fisch et al have reportedly identified these PDEs in human spermatozoa and they suggest they play a role in the acrosome reaction and motility respectively (Fisch et al., 1998). The expression of PDE 1 and 4 has also been reported in mouse spermatozoa (Yan et al., 2001, Geremia et al., 1984, Rossi et al., 1985) and rat spermatozoa (Geremia et al., 1982, Naro et al., 1996). Using the inhibitor of PDE3 and PDE5 (Milrinone and Sildenafil), Lefièvre et al. has also suggested

the presence of these PDE's in ejaculated human spermatozoa; although PDE5 was said to be present at very low levels (Lefièvre et al., 2000, Lefièvre et al., 2002a).

Although it is known that cyclic nucleotides, such as cAMP, are essential for sperm functions, the concept of modulating sperm cAMP level through inhibition of PDE activity are recently being explored for the treatment of male factor infertility. Pentoxifylline (PTX), a non-specific inhibitor of PDE, has been used clinically to stimulate human sperm motility. In a clinical study (Yovich et al., 1990), a strong correlation between PTX and fertilization rate was observed. Using spermatozoa pre-incubated (30min) with PTX prior to insemination, a significant increase in fertilization rate was observed; 20.4% in control group vs 47.4% in PTX treated group (Yovich et al., 1990). Also, when PTX was added to sperm cells during IUI sperm preparation in a group of asthenozospermic and oligoasthenozoospermic patients (101 cycles) a significant ($p<0.05$) increase in pregnancy rate was observed in PTX treated group (27.5%, 40 cycles) in comparison to control group (11.5%, 61 cycles) (Negri et al., 1996). Nevertheless, the concentration required to induce a motility enhancing effect (1-10mM) has raised concern regarding the use of non-specific PDE inhibitors. At this concentration range, others have also reported that the use of non-specific PDE inhibitor enhances induced acrosome reaction (Tasdemir et al 1993). Since acrosome reaction represents an important step in sperm-oocyte interaction, premature induction is considered detrimental; the fertilizing ability of sperm decreases when the acrosome reaction has taken place prior to oocyte penetration, even if there is enhancement of motility as a result of PTX treatment (Buffone, et al 2008).

Finally, since inhibition of PDEs still represents a potential mechanism by which sperm cellular processes can be modulated, identification of PDE inhibitor with type specificity is of crucial importance in order to eliminate the possibility of other PDEs being inhibited and in turn causing detrimental effects.

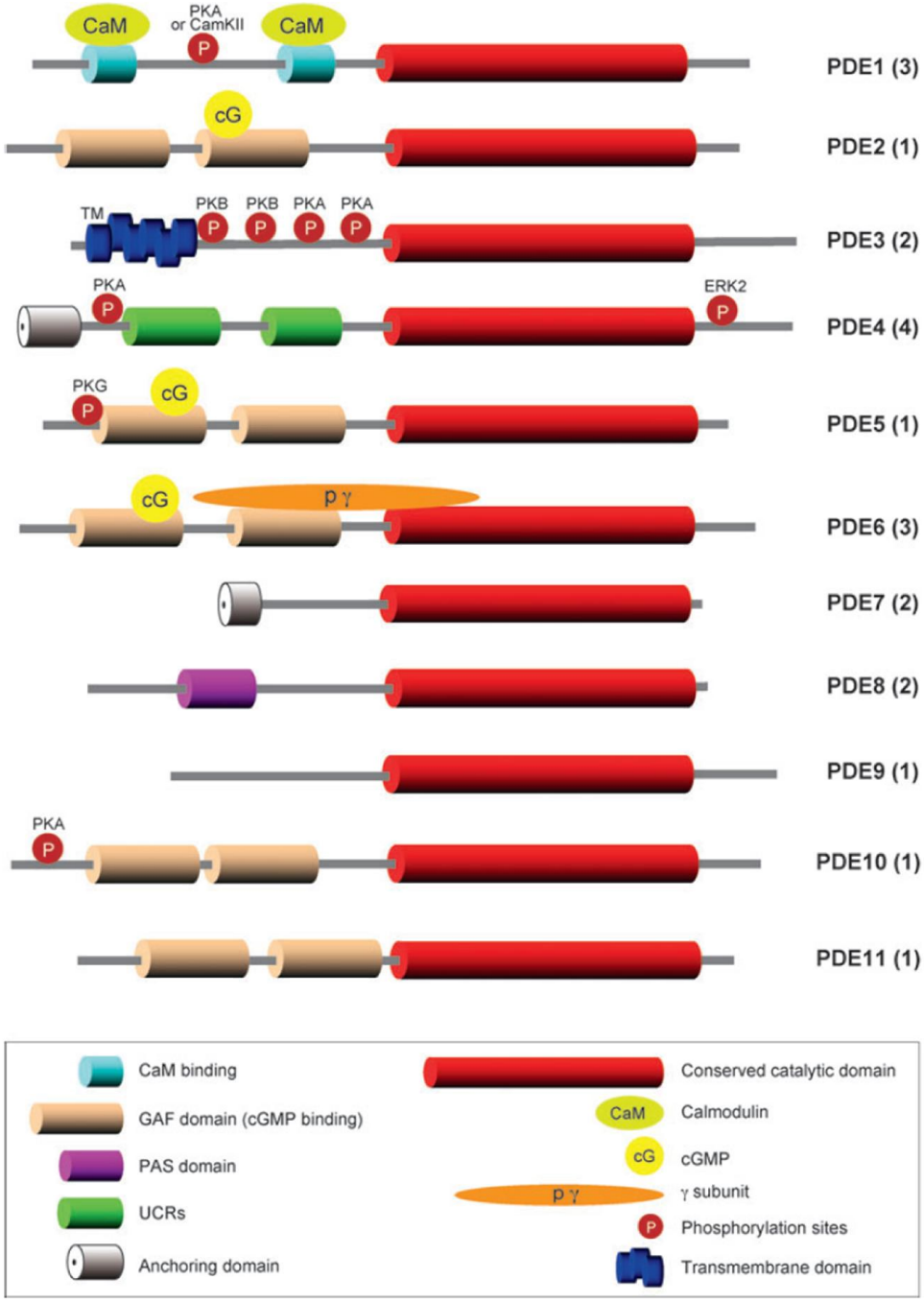


Figure 1-4: Schematic representation of the structure of the 11 cyclic nucleotide phosphodiesterase (PDE) families. The family name is reported on the side of each structure, and the number in parenthesis denotes the number of genes composing the family (Francis et al., 2011).

1.3.3.3 PKA and Tyrosine Phosphorylation

A consequence of increase in intracellular cAMP level is the activation of cAMP-dependent kinase (PKA). The presence and activity of PKA has already been reported in bovine (Garbers et al., 1973), mouse (Visconti et al., 1997) and human sperm (Lefièvre et al., 2002b). Its activity has been reported to be crucial in almost every aspect of sperm function that has been analysed so far (Baker, 2011, Taylor et al., 2004). The activation of PKA holoenzyme occurs due to cAMP binding to the regulatory subunit of PKA and this leads to dissociation of the catalytic (C) subunits. Two isoforms (type I and type II) of this holoenzyme are present in sperm cells and type II isoforms are the dominant form of these isoenzyme in elongating spermatids while type I are considered to be selectively decreased during spermatids development (Landmark et al., 1993, Oyen et al., 1990). RII holoenzyme is considered to be held firmly to the flagellum by the A kinase anchoring proteins (AKAPs) that serve as scaffolds for PKA. AKAPs localise PKA to specific sites in the cell by docking to the N-terminus of the regulatory subunits, thus creating microenvironments for PKA signalling that allows for rapid but selective phosphorylation and dephosphorylation of targeted proteins (Burton et al., 1999, Taylor et al., 2004).

However, a study by Burton et al. 1999, produce data to suggest that delocalization of PKA by disrupting the RII_{α} subunit of PKA does not affect mouse sperm motility or fertilizing ability. This result is rather unexpected, because one would have thought delocalization of PKA would lead to erratic activation which might compromise motility or fertility (Burton et al., 1999). The catalytic subunits of PKA have been, nonetheless, demonstrated to be important for the enzymatic activity of PKA. Using $PKA_{C\alpha}$ knockout mice, (Skalhegg et al., 2002), showed that these mice had little to no PKA activity in their sperm and although they have normal spermatogenesis there is defective progressive motility. Since this observation implies that $PKA_{C\alpha}$ is the key to the kinase activity of PKA, other catalytic subunits of PKA

have been probed. PKAαII, a catalytic subunit, among others; has been identified to be responsible for triggering hyperactivated motility by affecting downstream pathways which involve up-regulation of tyrosine phosphorylation in the flagellar region of the sperm cell (Nolan et al., 2004b, Baker et al., 2009). PKAαII knockout mice are infertile and this is consistence with the presume significant role of hyperactivated motility in penetration of the oocyte, in addition; no increase in tyrosine kinase cascade was observed in the PKAαII knocked out mouse sperm (Nolan et al., 2004b, Baker et al., 2009).

Although there is consensus that PKA activity is associated with increase in protein tyrosine phosphorylation (Leclerc et al., 1996a, Leclerc et al., 1996b, Visconti et al., 1995a, Aitken et al., 1998a), the events occurring between the early activation of PKA (Lefièvre et al., 2002b, Visconti et al., 1997) and protein tyrosine phosphorylation are not fully elucidated. As PKA is a serine/threonine kinase, it cannot play a direct role in tyrosine phosphorylation and therefore must activate tyrosine kinases indirectly (Leclerc et al., 1996a). Studies by Baker et al., 2006 and 2009 have implicated two intermediate tyrosine kinases (SRC and c-Abl) that respond to PKA activation (Baker et al., 2006, Baker et al., 2009). Both SRC and c-Abl have been localised within mouse sperm flagellum, where most capacitation-dependent tyrosine phosphorylation occurs (Baker et al., 2006, Baker et al., 2009). Additionally, as cells became hyperactivated, autophosphorylation on SRC and c-Abl could be detected in the sperm tail and this event could be suppressed with the PKA inhibitor, H89 (Baker et al., 2009). However, with the complexity of the cAMP induced tyrosine phosphorylation, that is (1) response in terms of the subcellular structures phosphorylated (fibrous sheath, axoneme, mitochondrial membranes) and (2) the differential timing of these events, the involvement of more than two species of intermediate tyrosine kinase cannot be ruled out (Lin et al., 2006, NagDas et al., 2005, Petrunkina et al., 2003).

1.4 Hyperactivation

First observed in hamster sperm (Yanagimachi 1970), hyperactivation (hyper-activated motility) is characterised by a high amplitude, asymmetrical flagellar waveform. This form of motility pattern has been observed in all eutherian mammalian sperm examined, including humans (Burkman, 1984). In most species, these waves develop only on one side of the tail, producing asymmetrical flagellar beating and circular or figure-of-eight swimming trajectories. Hyperactivation can be observed in situ in transilluminated murine oviducts around the time of ovulation and near the site of fertilization (Suarez and Osman, 1987, Suarez, 1987). However, hyperactivation has not been observed directly within the human oviduct and detailed reports on the swimming patterns of sperm recovered from the human oviduct are lacking. Hyperactivated sperm are more efficient at penetrating highly viscoelastic media and because sperm do encounter mucus in the oviduct and must also pass through the matrix of the cumulus oophorus and the zona pellucida, it is believed that this form of motility enables sperm to serves this key function of penetration in vivo (Suarez and Pacey, 2006, Pacey et al., 1995). Mouse sperm have been observed to use the deep flagellar bends exhibited during hyperactivated motility to turn around within pockets of mucosa and escape out into the central lumen (Suarez and Osman, 1987). Also in mouse, only sperm exhibiting hyperactivated movement in the oviduct were observed to detach from the epithelium (Demott and Suarez, 1992). Human sperm observed detaching from oviductal epithelium in vitro showed a greater incidence of hyperactivation than sperm that had not yet attached to epithelium (Pacey et al., 1995)

1.4.1.1 Role of cAMP/PKA and Calcium ion in Hyperactivation

Despite the importance of hyperactivation to fertilization, little is known of the biochemical pathways regulating its expression. The majority of what is known about protein targets that are involved in hyperactivation are identified while using demembranated sperm cells. There

is evidence that cAMP pathways are involved in signalling the onset of hyperactivation (White and Aitken, 1989, Yanagimachi, 1994). Treatment of sperm cells with membrane permeable analogues of cAMP or phosphodiesterase inhibitors has been found to enhance hyperactivated motility in hamster (White and Aitken, 1989); human (Calogero et al., 1998), and monkey sperm cells (Yeung et al., 1999) in vitro. In a recent study, by Yu et al, the importance of cAMP pathway in hyperactivation was further emphasized. Male rats that had their *sAC* knockdown by electroporation-mediated shRNAs showed a significant reduction in hyperactivation rate. Thus, interfering with sAC expression indirectly regulated the level of sperm protein phosphorylation and in turn reduced hyperactivation rate was observed (Yu et al., 2013).

Additionally, it has been demonstrated that Ca^{2+} signalling involving extracellular Ca^{2+} is critical for hyperactivation. When Ca^{2+} blockers were added to hyperactivated hamster sperm to inhibit hyperactivation, most sperm failed to penetrate the zona even though they remained motile and underwent acrosome reactions (Stauss et al., 1995). Increased cytoplasmic concentrations of free Ca^{2+} have also been detected in the flagellae of hyperactivated sperm (Ho and Suarez, 2001, Suarez et al., 1993). Put together, this indicates that the predominant source of Ca^{2+} for hyperactivation is through extracellular source. Indeed, male mice that are null mutants for CatSper-1, -2, -3 or -4, the main transmembrane channel that transports Ca^{2+} into the intracellular domain, are infertile and the infertility has been correlated to a failure to hyperactivate (Ren et al., 2001, Jin et al., 2007, Qi et al., 2007, Quill et al., 2003)

Demembranated sperm have been used to identify calmodulin (CaM) as the intermediate messenger protein that transduces calcium signals during hyperactivation. When calmodulin was extracted from bull sperm during demembranation, motility was not reactivated unless exogenous CaM was added back to the cell. Addition of 1μM Ca^{2+} with CaM causes hyperactivation in reactivated demembranated sperm. When peptide inhibitors of calmodulin

kinase II (CaMKII) were added with the CaM, hyperactivation was reduced by 75%; whereas, W-7 or a peptide inhibitor of myosin light chain kinase added with the CaM did not inhibit hyperactivation. When KN-93, an inhibitor of CaMKII, was added to intact sperm, caffeine-induced hyperactivation was inhibited without impairing normal motility. CaM and CaMKII were immunolocalized to the flagellum in bull and mouse sperm (Ignotz and Suarez, 2005, Schlingmann et al., 2007). A study by Marin-Briggiler identified the presence of CaMKIV in the flagellum of human sperm using a monoclonal antibody, however, monoclonal antibody against CaMKII did not detect its presence in human sperm (Marin-Briggiler et al., 2005). It has to be noted, however, that a non-detection of CaMKII as well as CaMKI cannot completely negate their presence in human sperm.

1.5 Sperm-Oocyte Interaction

The key purpose of capacitation is to ensure that spermatozoa are in the appropriate state to fertilize oocytes, by finely controlling the rate of the changes necessary to prime spermatozoa and by activating all the mechanisms needed for the acrosome reaction. The acrosome reaction (AR) is an exocytotic event that is considered to be essential for fertilization (Yanagimachi, 1994). During initiation of the acrosome reaction, a sperm's plasma membrane fuses with the underlying outer acrosomal membrane leading to their fenestration and vesiculation, to release the acrosomal contents (Yudin et al., 1988, Yanagimachi, 1994). The release of the acrosomal contents, coupled with the thrust provided by the hyperactivated beating of its flagellum enables a sperm to penetrate the ZP (Yanagimachi, 1994). Although the release of the acrosomal content is crucial to fertilization, because sperm that fail to initiate the acrosome reaction cannot penetrate the zona pellucida and hence are denied access to the egg plasma membrane (Yanagimachi, 1994), there is debate as to when the acrosome reaction actually takes place during sperm-oocyte interaction.

For many years it was believed that binding of sperm to the zona pellucida triggers acrosome reaction; consequently only sperm cells reaching the egg with an intact acrosome are able to fertilize an oocyte (Florman and Storey, 1982, Gupta and Bhandari, 2011, Saling and Storey, 1979). On the contrary, several studies have also suggested that AR occur before binding to the ZP in several mammalian species (Bedford, 2011). In a recent studies using mice as a model (Jin et al., 2011, Inoue et al., 2011), it was shown that most fertilizing mouse spermatozoa begin their AR prior to making contact with the ZP during in vitro fertilization. Additionally, acrosome reacted spermatozoa recovered from the perivitelline space of mice were able to fertilize other oocytes (Inoue et al., 2011). These observations challenge the model that suggested that acrosome intact sperm needs to bind to the ZP and then acrosome react before they can fertilize an oocyte. However, it is important to state that the physiology of sperm-oocyte interaction may be species-specific and what applies to mice may not be the same for human gametes, therefore, further studies are required to determine the exact role of the zona pellucida on the acrosome status of human sperm and when exactly the acrosome reaction should take place in order to achieve fertilization.

The primary *in vivo* initiator of AR is believed to be a glycoprotein constituent of ZP, designated ZP3 (or ZPC) (Wassarman, 1999, Kopf, 2002), although a number of other endogenous agents including progesterone (Osman et al., 1989), prostaglandin (E1) (Schaefer et al., 1998) and acetylcholine (ACh) (Bray et al., 2002) have been identified to be capable of triggering acrosomal exocytosis *in vitro*. Induction of AR by these endogenous agents leads to a rapid increase in intracellular calcium. ZP3 induce AR by activating non-selective cation channels that lead to membrane depolarization and opening of voltage-sensitive Ca^{2+} channels (Florman et al., 1992, Florman et al., 1989). Similar to ZP3, progesterone can induce acrosome exocytosis in human sperm, however, this effect is dependent on the presence of extracellular calcium (Blackmore et al., 1990, Blackmore et al., 1991). Calcium

has been suggested to directly play a role in the fusion events in sperm membrane. Using ram spermatozoa, Watson et al., 1995 studied the temporal and spatial location of intracellular calcium during acrosome reaction. Calcium was identified by a pyroantimonate-osmium fixation technique and as fusion developed, Ca^{2+} was evident on the outer acrosomal membrane. At later stages, Ca^{2+} is localised in both post acrosomal dense lamina and on outer acrosomal membrane under the equatorial segment (Watson et al., 1995). These findings suggest that Ca^{2+} may be implicated in the fusion process.

In addition to Ca^{2+} influx, activation of protein kinases (such as PKA) has been reported during sperm acrosome reaction (Lefièvre et al., 2002b). Evidence supporting the involvement of PKA during AR also demonstrates increase in adenylyl cyclase activity and cAMP production during this process. When capacitated spermatozoa were pre-treated with inhibitors of PKA (KT5720), PKC (Calphostin C) and PKG (KT5823), before induction of acrosome reaction by solubilized ZP, a significantly lowered percentage of acrosome reacted cells was observed in comparison to control (Bielfeld et al., 1994). Also, no significant stimulation of the acrosome reaction occurred when kinase stimulators were added to spermatozoa which had been pre-treated with inhibitors of the kinases (Bielfeld et al., 1994). These findings suggest that protein kinases may be involved in AR. Thus, any compound(s) that increases the intracellular availability of cyclic nucleotides may indirectly induce acrosome reactions since increase in cyclic nucleotide will lead to increased protein kinase activity.

Indeed, treatment of sperm cells with some PDE inhibitors have been suggested to promote acrosome reaction (Fisch. et al., 1998). Using 8-MeOM-IBMX, a PDE 1 inhibitor, Fisch and colleagues reported a significant stimulation of sperm acrosome reaction in human sperm. However, in the same study, Rolipram (a PDE4 inhibitor) was found to induce a significant improvement in sperm motility but in contrast to 8-MeOM-IBMX, Rolipram did not affect

the acrosome (Fisch et al., 1998). Nonetheless, there is conflicting data regarding the effect of Sildenafil (a PDE 5 inhibitor) on inducing the acrosome reaction. In 2000, Lefièvre, et al reported that Sildenafil increases sperm motility, capacitation, protein tyrosine phosphorylation but not the acrosome reaction, in contrast, Glenn et al demonstrated that Sildenafil induced a significant increase in the percent of acrosome reacted sperm (Lefièvre et al., 2000, Glenn et al., 2007). From these results, it can be concluded that differential activation by type-specific phosphodiesterase inhibitors might account for the observation recorded in these studies (Fisch et al., 1998). In addition, since PDE1 is predominantly localised in the sperm head (Lefièvre et al., 2002a) the effect of 8-MeOM-IBMX in relation to the induction of acrosome reaction will make sense. In view of the potential to induce acrosome reaction, it is important to evaluate the effect of PDE inhibitors in order to determine if they affect acrosomal status of human sperm cells.

1.6 Computer Assisted Motility Analysis

With the development of computer assisted sperm analyser (CASA), the movement characteristics of hundreds of spermatozoa can be objectively assessed, in real time, within minutes. With negative phase contrast optics, the system identifies sperm in the field of view based on the pertinent settings of sperm head size (length and width) and illumination (Mortimer, 1997). Motility parameters are measured by tracking the sperm head as opposed to the flagellum; because head movement reflects the beating of the flagellum. For each spermatozoon tracked, the system reconstructs a trajectory using the centremost point or the brightest region of the sperm head as a reference point and this generates huge information on series of kinematic values. The kinematic values proffers information about the distance travelled in within a set time period, and is therefore a direct reflection of sperm velocity (Figure 1-5).

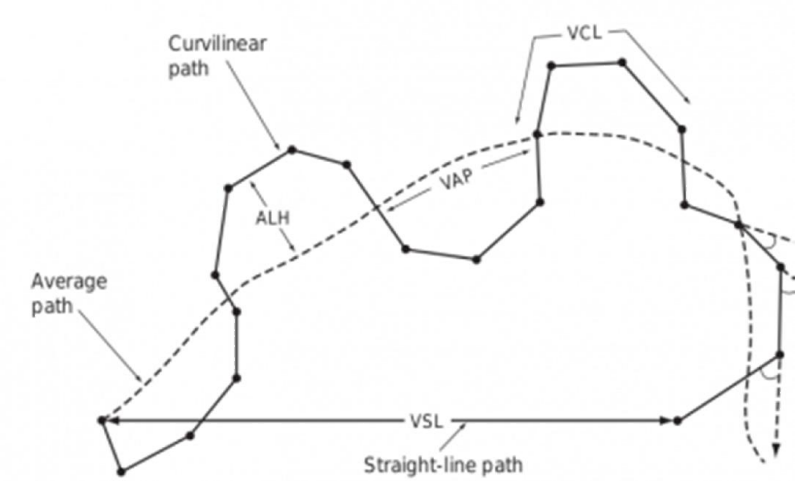


Figure 1-5: Schematic representation of the different velocities as measured by the computer assisted sperm analysis (CASA) systems. VSL = straight-line velocity, VAP = average path velocity, and VCL = curvilinear velocity. See text for more explanation concerning these parameters.

The curvilinear velocity (VCL) is a measure of the overall distance that the sperm head travelled in any direction. This is dependent on the frequency of tail beating, and the wavelength and amplitude of the flagellar movement (Suarez and Dai, 1992). Average path velocity (VAP) is the length of the general trajectory, that is, the distance the sperm head travels in the average direction of its movement (Mortimer, 2000). The straight-line velocity (VSL) is the shortest distance between the start and end of the sperm trajectory; it therefore describes the displacement of the sperm (Mortimer, 2000). Using the ratio of the velocity values, linearity (LIN): (VSL/VCL) x 100, and straightness (STR): (VSL/VAP) x 100, additional information that further enhances the distinction between types of motility is generated by the CASA system. The amplitude of lateral head displacement (ALH) is the width of the side to side movement of the sperm head and it largely corresponds to the degree of bending initiated at the sperm neck (Oehninger and Kruger, 2007). Lastly, combination of VCL, ALH and LIN is used to define cells with hyperactivated motility. For human

spermatozoa, cells analysed at 60Hz and with VCL ≥150μm/s, ALH ≥7μm and linearity ≤50% are categorised as hyperactivated spermatozoa (Mortimer et al., 1998).

Although CASA systems have their limitations, because cells swimming too tightly are difficult to track, it has distinct advantage over manual (eye) motility assessment. As mentioned above, it can determine cell velocity with high degree of accuracy, which is impossible to achieve by eye, and assessment of hundreds of spermatozoa in less than a minute is an added advantage over old methods, such as video micrograph; which are time consuming and laborious. Numerous investigators have also addressed whether or not CASA measurements could be useful in providing prospective indications regarding sperm fertilizing capacity (Barratt et al., 1993, Irvine et al., 1994, Larsen et al., 2000, Verstegen et al., 2002). In a study by Donnelly and colleagues, sperm velocity measurements of human spermatozoa were shown to correlates with penetration of zona-free hamster eggs and human oocytes in vitro (Donnelly et al., 1998). In addition, VCL, VAP and VSL values were reportedly higher in samples that achieved ≥50% fertilization compared with ≤50% fertilization. In another study investigating the relationships between CASA estimates and fertilization rates in vitro, Hirano and colleagues showed that there were significant differences in VCL ($p < .005$), average path velocity (VAP) ($p < .005$), and Rapid ($p < .05$) between prepared samples (swim-up) of 108 “good” and 28 “poor” fertilization groups (Hirano et al., 2001). In conclusion, CASA systems have high levels of precision and reliability and they are a great tool to objectively compare sperm motility (based on different treatment conditions) and to improve our knowledge and ability to manipulate spermatozoa.

1.7 Aims and Objectives

The aim of this study is to examine the effect of second generation phosphodiesterase inhibitors on sperm functionality. The main hypothesis is that phosphodiesterase are involved in regulation of sperm functions and by inhibiting phosphodiesterase activity sperm functions, such as motility, can be modulated. In the first set of experiments, 43 compounds with reported phosphodiesterase inhibitor activity will be screened. Identified compounds will then be subjected to further testing with specific reference to (1) Effects on quantitative motility, including assessments of penetration into artificial cervical mucus substitutes (2) The potential negative effects of these compounds e.g. induction of the acrosome reaction (3) Effects of these compounds on patients samples under clinical conditions of treatment. . It is hypothesised that by using a two phase drug discovery screening approach compounds that have motility enhancing effects on human spermatozoa, without affecting other sperm function negatively, can be identified. In the second set of experiments, studies will be done to investigate the effects of promising compounds on cyclic nucleotides levels and the potential biological pathway effecting enhancement of motility. Finally, an animal model will be developed to assess if increase in motility translates to higher fertilization rate, and high performance liquid chromatography (HPLC) techniques will be optimised for routine detection of cyclic nucleotides in sperm cells

Chapter 2.

Materials and Methods

2.1 Ethical Approval

Written consent was obtained from each patient (APPENDIX 3) in accordance with the Human Fertilization and Embryology Authority (HFEA) Code of Practice (version 8) under local ethical approval (08/S1402/6) from the Tayside Committee of Medical Research Ethics B. Similarly, volunteer sperm donors (healthy men randomly selected from the general public [usually students] with no known fertility problems) were recruited in accordance with the HFEA Code of Practice (version 8) under the same ethical approval.

2.2 Semen Samples

Semen samples from donors and patients were collected by masturbation into a sterile plastic container after 2-4 days of sexual abstinence. The samples were used for analysis after liquefaction of the semen at 37°C for approximately 30 minutes and within 1 hour of production. Normozoospermic donors were recruited for this study after an initial semen analysis based on W.H.O. 2010 criteria for sperm concentration (≥ 15 million/ml) and motility (≥ 40% total motility and ≥ 32% progressive motility) (W.H.O., 2010). Semen samples obtained from patients were assessed for the semen profile by clinical embryologists according to W.H.O. criteria (W.H.O., 2010). Patients were selected for IVF or ICSI according to clinical indications and semen quality.

2.3 Media

A modified Synthetic tubal fluid (Mortimer, 1986) was used as **capacitating media** (CM): 1.8 mM $CaCl_2$,5.4 mM KCl, 0.8 mM $MgSO_4.7H_2O$,116.3 mM NaCl, 1.0 mM NaH_2PO_4, 5.55 mM D-glucose, 2.73 mM sodium pyruvate, 25 mM sodium lactate, 26 mM sodium bicarbonate and 3mg/ml BSA. The **non-capacitating medium** (NCM) was similar to CM but without sodium bicarbonate (NCM): 1.8 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgSO_4.7H_2O$,

116.3 mM NaCl, 1.0 mM NaH_2PO_4, 5.55 mM D-glucose, 2.73 mM sodium pyruvate, 41.75 mM sodium lactate, 25 mM HEPES and 3mg/ml BSA. BSA was added to NCM to prevent cells from sticking on the glass slides when taking motility reading on CASA. The osmolality of buffers was adjusted to 290-320 mOsm/kg and pH 7.4 was adjusted using 1M NaOH solution. **TBST** (Tris-Buffered Saline and Tween 20) consists of 20 mM Tris, 136 mM NaCl and Tween20 (0.1% final conc.). **Swim-up media** was composed of 97.8mM NaCl, 4.69mM KCl, 0.2mM $MgSO_4$, 0.37mM KH_2PO_4, 2.04mM $CaCl_2$, 21mM HEPES, 21.4mM Lactic acid, 2.78mM glucose and 0.33mM Na-pyruvate.

2.3.1 Density Gradient Centrifugation (DGC)

2.3.1.1 Donor Sperm Preparation

Semen samples were produced at home after 2-4 days of sexual abstinence and collected in sterile 60ml containers. The samples were used for analysis after liquefaction of the semen at 37°C for approximately 30 minutes and within 1 hour of production. An initial assessment of sperm concentration and motility was carried out in order to determine the quality of the individual semen sample using CASA by placing 4µl of the raw sample on a 20µm depth Hamilton-Thorne slide with a 22mm by 22mm coverslip. Two different sperm populations were isolated using a 40-80% discontinuous density gradient procedure. Maximally 2 ml of semen was loaded after 30 min of liquefaction at 37˚C on the top of a colloidal silica suspension (Percoll) made of 80% and 40% layered (2ml each). The density gradient was centrifuged at 300g for 20 min at room temperature. The high quality cells form a pellet in the 80% fraction and the poor motility population are at the interface of 40-80%; respectively called 80% and 40% fraction. Depending on experiment to be undertaken, either the 40% fraction or the pellet (80% fraction) was then taken by a Pasteur pipette and placed into 15ml conical bottom falcon tube containing 5mls of NCM. Sperm cell fractions were washed in NCM by centrifugation for 10 min at 500g and the sperm pellets were resuspended using a

NCM or CM at ~20-25x10^6 cells/ml. The cells resuspended in CM are incubated at 37^{O}C in a 5% CO_2 humidified atmosphere for 3hrs in order to initiate sperm capacitation (Mansour et al., 2008, Giojalas et al., 2004).

2.3.1.2 Patient Sperm Preparation

Patient samples were produced at the Assisted Conception Unit (ACU) and allowed to liquefy in a 37°C incubator within 30 minutes allowing the sperm to become freely motile. Sperm preparation for IVF/ICSI/IUI treatment was carried out at the ACU by an embryologist and any sample surplus to clinical use (Raw semen sample or IVF/ICSI sample used in patient treatment) was used in this study. An initial assessment of sperm concentration and motility (as above) were carried out to determine the quality of individual samples. The samples were prepared as for donor samples, however commercially available media was used for sperm preparation. The 40-80% discontinuous density gradients were made using PureSperm (Nidacon, Mölndal, Sweden) diluted with Cook Sydney IVF Gamete Buffer, a HEPES buffered solution (Cook Sydney IVF Limited, National Technology Park, Ireland, UK). Samples were centrifuged at 300g for 20 minutes. The supernatant was discarded, and the pellet washed by centrifugation at 500g for 10 minutes in 4 ml of Cook Gamete Buffer. The pellets were resuspended in media depending on whether they were for IVF or ICSI/IUI. IVF samples were suspended in pre-equilibrated 1ml Cook Sydney IVF Fertilization medium (IVF FM), a bicarbonate buffered medium similar to CM (see media above) and ICSI/IUI samples were resuspended in Cook Gamete Buffer similar to NCM (see media above). IVF samples were then incubated at 37°C with 6% CO_2 for at least 3 hours in order for the samples to capacitate whereas ICSI samples were left at room temperature until the time for injection procedure. Following insemination (IVF) or injection (ICSI) the remaining portion of the sample was analysed for change in motility and kinematic parameters as a result of compound treatment.

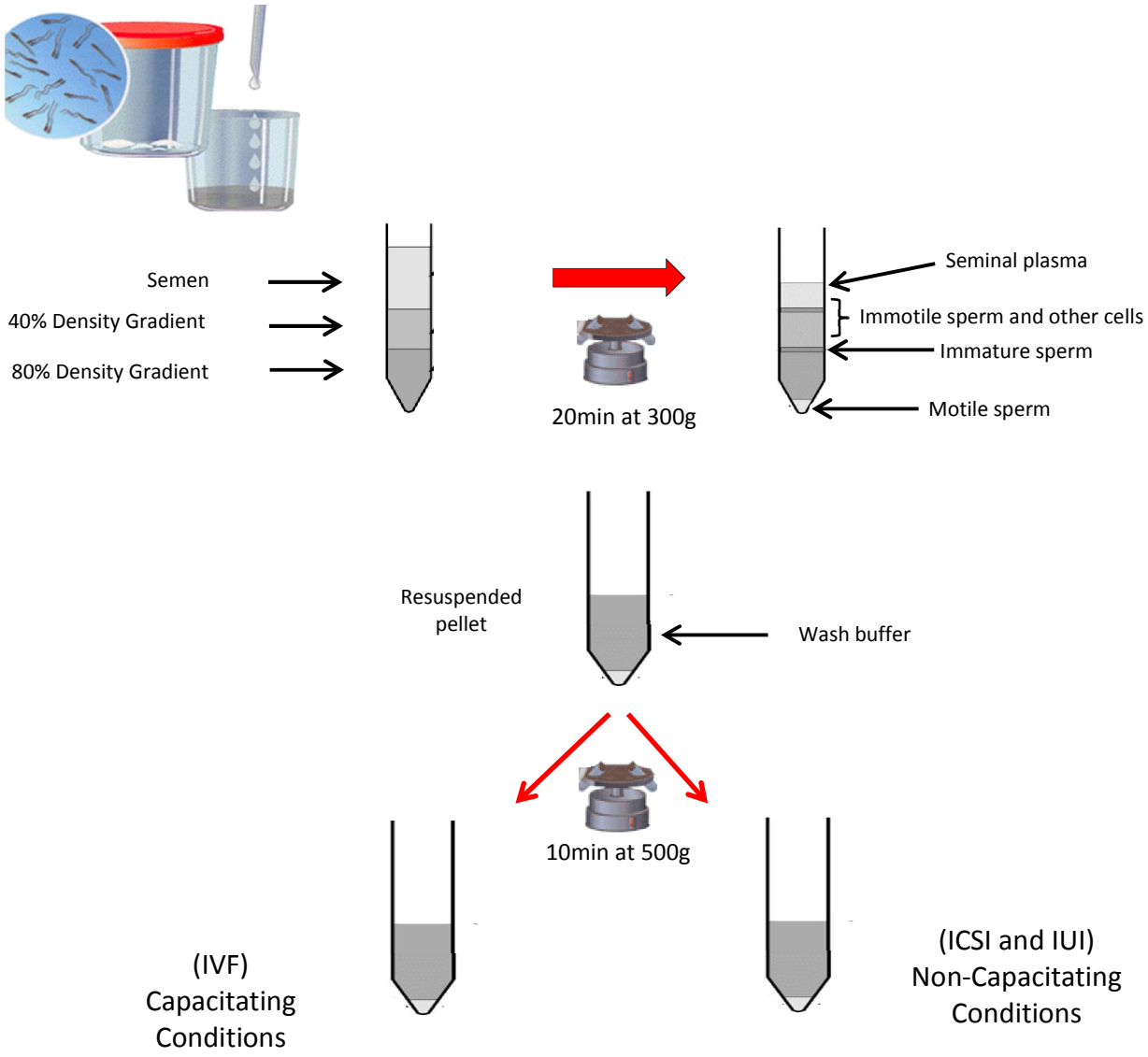


Figure 2-1: Density gradient preparation: Semen samples were collected by masturbation into a sterile plastic container after 2-4 days of sexual abstinence. The samples were allowed to liquefy for approximately 30 minutes at 37°C. After liquefaction, 2 ml of semen was loaded on top of a colloidal silica suspension (Percoll) made of 80% and 40% layered (2ml each). The density gradient was centrifuged at 300g for 20 min at room temperature. Depending on experiment, either the 40% fraction or the pellet (80% fraction) was then taken by a Pasteur pipette and placed into 15ml conical bottom falcon tube containing 5mls of NCM (wash buffer). Sperm cell fraction were washed in NCM by centrifugation for 10 min at 500g and the sperm pellet were resuspended using a NCM or CM

2.3.2 Direct Swim-Up

Direct swim-up technique was used to isolate motile spermatozoa on the basis of their ability to swim out of seminal plasma into culture medium. 1ml of liquefied semen was gently dispensed at the bottom of a 50ml falcon tube containing 5ml of HEPES buffered swim-up media (see 2.3 above for media composition). To increase the surface area of the interface between the semen layer and the culture medium, tube was incubated at 45^{o} angle for 1hr at $37^{o}C$ in a non- CO_2 incubator. After incubation, in order not to contaminate the interface, 3ml of the top layer was removed into a new 15ml falcon tube. This was then left at room temperature for 1-2hr to form a pellet; and the isolated spermatozoa were resuspended in capacitating media or non-capacitating media respectively.

2.4 Assessment of Sperm Motility

Sperm kinematic were analysed using CASA Hamilton-Thorne HTM-CEROS (version 12), which provides individual kinetic characteristics of tracked spermatozoon. At the end of each incubation or treatment, aliquot (3µl) of samples were loaded onto pre-warmed 20µm depth chamber slide (Conception Technologies) that allow lateral head displacement of spermatozoa. This was then placed onto MiniTherm stage warmer ($37^{o}C$) attached to a standard (negative) phase contrast microscope under a low field illumination with x10 objective. System parameter settings for these analyses were 30 frames at 60 frames per second (Hz); minimum contrast 80; minimum size 3 (pixels); upper and lower gates of 0.39 and 1.4 for intensity, and 0.85 and 4.24 for size, and the default values for non-motile cells were 6 pixels and 160 for size and intensity respectively. To ensure error was reduced during data acquisition, the playback option was used after each analysis to check cell trajectory analysed. Sixteen randomly selected microscopic fields, from two sections of the chamber

slide, were examined for each sample. Spermatozoa with average path velocity (VAP) > 25μm/s and straightness (STR) ≥80% are considered progressive while those with curvilinear velocity (VCL) >150 μm/s, amplitude of lateral head displacement (ALH) >7.0 μm, and decrease in linearity [(VSL/VCL) x100] LIN <50% are defined as hyperactivated (Mortimer, 2000, Mortimer et al., 1998).

2.5 Statistical Analysis

Statistical analysis of the effects of different compounds was conducted using analysis of variance (ANOVA), and t-test using SPSS 20.0 (Statistical Package for Social Science) software, a *p*-value <0.05 was considered significant. Kolmogorov-Smirnov test was used to confirm assumption of normality. Data that were not normally distributed were transformed. Homogeneity of variance was tested using Levene's test.

Chapter 3.

Identification of compounds with clinically relevant enhancement of human sperm motility

3.1 Introduction

The functional characteristic of spermatozoa is their motility capabilities that allow them to penetrate cervical mucus, migrate through the uterus into the fallopian tube and to penetrate the oocyte. Among other key semen parameters, such as sperm concentration and morphology, sperm motility significantly correlates with fertilization rate in vitro (Liu et al., 1991, Aitken et al., 1982). In addition, it is one of the key criteria in deciding which type of assisted reproductive technology should be performed. Impairment in sperm motility, sperm dysfunction, is the single most common cause of infertility (Irvine, 1998, Hull et al., 1985) and increasing number of couples are resorting to assisted reproductive technology in order to achieve a clinical pregnancy. Depending on the severity of sperm dysfunction, in male factor infertility, subjects are allocated to different forms of ART. In mild cases of sperm dysfunction, intrauterine insemination (IUI) is considered a more favourable and cost effective form of ART while in vitro fertilization (IVF) for moderate and intracytoplasmic sperm injection (ICSI) for severe case of sperm dysfunction (Van Voorhis et al., 2001, Barratt et al., 2011).

Currently, there is no clinically approved drug that can be added to spermatozoa in vitro or taken by a man in order to improve fertility. Since the early 1970's, however, many research groups have targeted different signal transduction mechanism regulating sperm motility so as to increase the fertilizing potential of sperm cells in vitro (Fakih et al., 1986, Hatakeyama et al., 2008, Wald, 2008). The cyclic AMP/protein kinase A (cAMP/PKA) pathway is now widely thought to have a role in the biochemical mechanism controlling sperm function with respect to regulation of motility. Most experimental evidences demonstrating a role for cAMP/PKA pathway in regulation of motility are based on observed increase in motility and concomitant elevation in intracellular cAMP after inhibition of cAMP catabolic enzymes by

phosphodiesterases inhibitors (Aitken et al., 1998b, Battistone et al., 2013, Baxendale and Fraser, 2005, Calogero et al., 1998, Leclerc et al., 1996a, Visconti et al., 2002). Non-specific phosphodiesterase inhibitors such as caffeine, 3-isobutyl-1-methylxanthine (IBMX), di-methylxanthine (theophylline) and pentoxifylline (PTX) have been shown to stimulate sperm motility and kinematic parameters in cases of severe male factor infertility (Rees et al., 1990, Yovich et al., 1990).

Following reports of motility enhancing effects of methylxanthines; clinical interest in the use of these compounds gained popularity in human assisted reproduction. A landmark study demonstrated a significant enhancement of fertilization rates when using non-specific PDE inhibitor (PTX) (Yovich et al., 1990) and 77 pregnancies were achieved (Yovich, 1993). Two IUI studies reported higher pregnancy rates following stimulation with PTX combined with IUI but the data were not part of a controlled study (Stone et al., 1999, Negri et al., 1996). In 1992, another study found both PTX and caffeine to significantly enhance sperm motility; however, detrimental effects on other sperm function (such as increased incidence of induced acrosome reaction) were reported (Tesarik et al., 1992). Since acrosome reaction is considered an important step in sperm-oocyte interaction, premature stimulation of the acrosome reaction is not a desirable effect and it counteract the motility enhancing benefits of these compounds. In view of the detrimental effects of non- specific PDE inhibitor and the widespread use of ICSI, which subsequently became the treatment of choice in male factor infertility, interest in sperm stimulation therapies for IVF/IUI has waned.

It is now clear that there are multiple forms of PDEs with different kinetic and regulatory properties and the regulation of intracellular cyclic nucleotides is therefore largely specified by the exact nature and localisation of the expressed PDEs (Francis et al., 2012). Also, human spermatozoa have been found to express several different PDEs and these are compartmentalised at different regions of the cells (Lefièvre et al.2002). Thus, using type-

specific PDE inhibitors that enhance motility without compromising other sperm function; it might be possible to overcome the limitations (e.g. premature stimulation of acrosome reaction) found when using non-specific PDE inhibitors. Surprisingly, in view of the plethora of existing and new-generation PDEIs (Francis et al., 2012) there are very few studies examining inhibition of specific PDEs on human sperm. Fisch and colleagues (Fisch et al., 1998) examined the biological activity of PDE1 and PDE4 in the sperm of 30 sub fertile men. PDE4 inhibition (via Rolipram) selectively increased the percentage of motile cells at 2 hrs and 24 hours of incubation and the most significant effect was noted on those samples with the lowest concentration of motile cells. PDE1 inhibitors (8-MeIBMX) selectively activated the acrosome reaction but this was not the case with inhibition of PDE4. The data on the role of PDE5 is controversial, probably because PDE5 represents only a very small fraction of the PDE activity (Lefieve et al., 2002). Biological effects have only been observed using relatively high concentrations of PDE 5 specific inhibitors. Induction of the AR (presumable due to increase [above a critical threshold] in cGMP) is frequently reported (Glenn et al., 2007).

3.2 Aims and Experimental Design

The aim of this chapter is to use drug screening approach to systematically and comprehensively screen a series of compounds with reported phosphodiesterase inhibitor activity, in order to identify key candidates that could be added to human spermatozoa in vitro to enhance sperm motility. To achieve this, the experiments were done in two phases. Phase 1 involves blind screening of 43 compounds with reported phosphodiesterase activity. Compounds that resulted in $\geq$ 60% increase in total motility were selected for further screening: motility and longevity, fertilizing ability and DNA/chromatin damage (Figure 3-1and Figure 3-2).

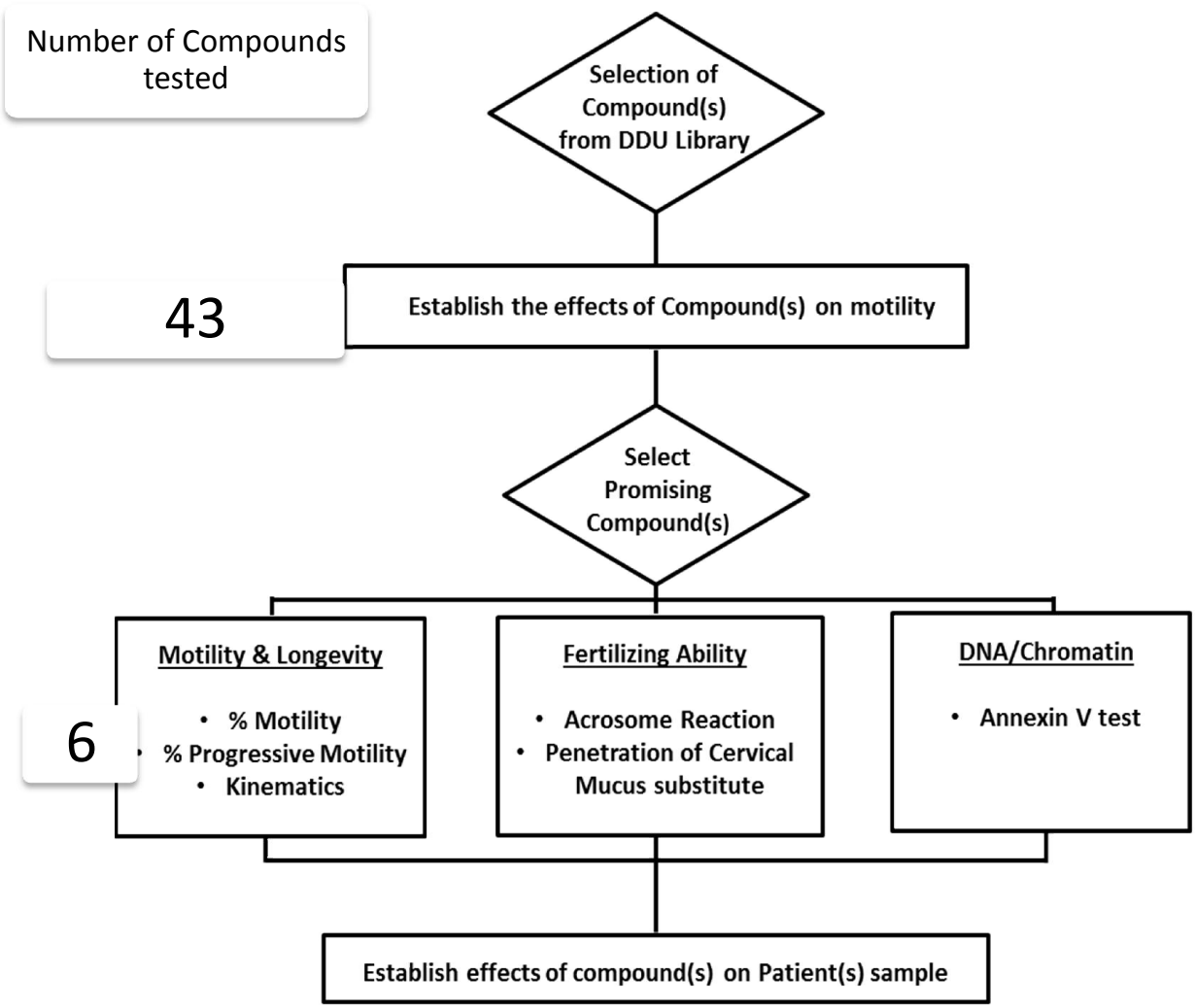


Figure 3-1 : Schematic diagram of overall experimental design: 43 compounds were identified and selected at the Drug Discovery Unit (DDU), University of Dundee. Effect of compounds on the kinematic parameter of human spermatozoa was assessed using CASA. Compounds that have robust and effective stimulation on sperm motility were selected for further testing to determine their effect(s) on sperm function.

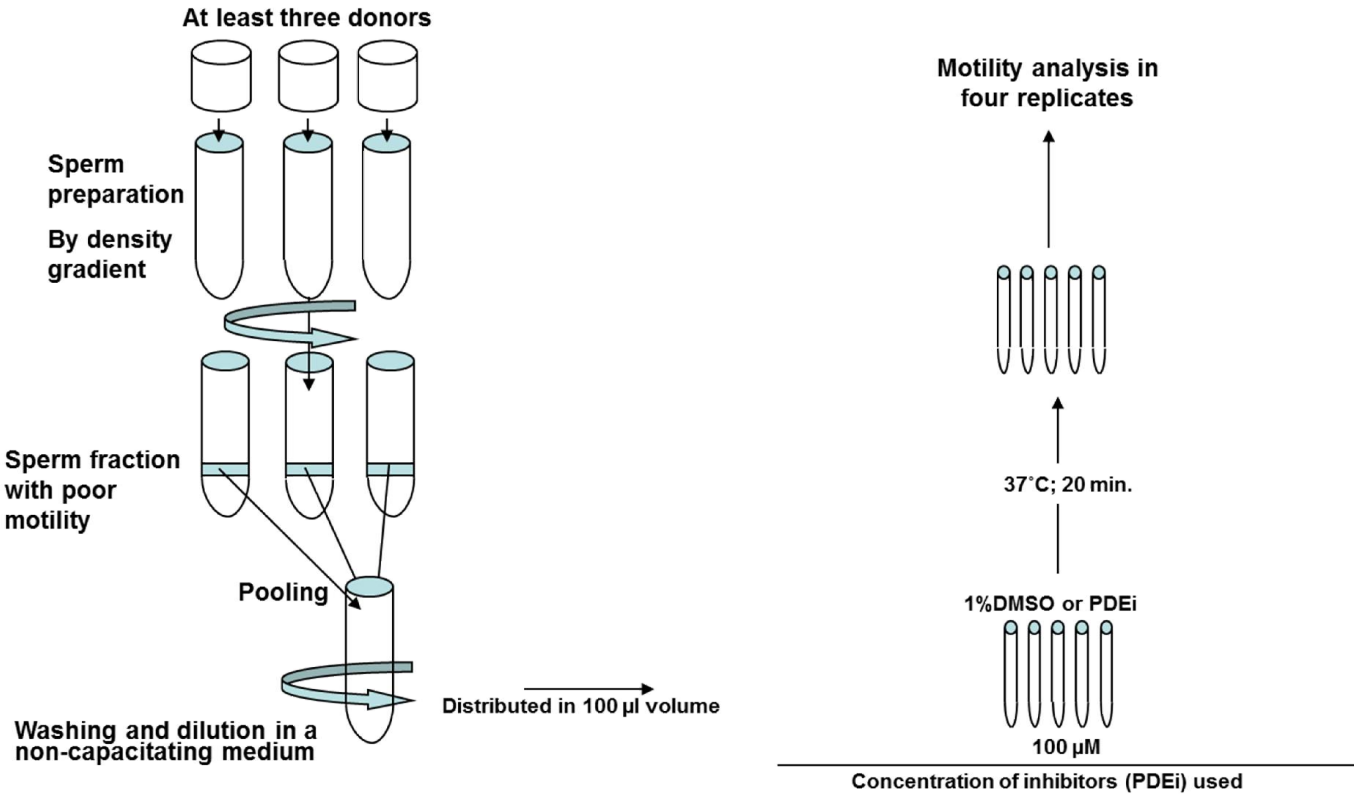


Figure 3-2: Procedure performed to screen different phosphodiesterase inhibitors in Phase 1. Three to four donors were used to isolate sperm cells with poor motility by density gradient centrifugation. Following the sperm preparation, spermatozoa were pooled together and 100 µl was used to assess quantitative sperm motility with 100 µM final concentrations of compound(s) for 20 min in NCM.

3.3 Experimental Procedures

3.3.1 Semen Preparation

The sperm samples were from healthy donors and the semen was prepared as described in Chapter 2.

3.3.2 Motility Analysis

Motility was recorded using CASA as described in Chapter 2.

3.3.3 Treatment of Spermatozoa with Compounds

In this study, forty three compounds (Table I) were initially tested before six were selected for further screening. The number of compounds tested at a given time was subject to the total volume of spermatozoa in suspension on a given day. Sperm sample (99µl) cultured under non-capacitating condition (including ICSI samples), were treated with 1µl (final concentration 100µM) of compounds and were incubated at 37^{o}C for 20min in a non- CO_2 incubator. However, for sperm under capacitating conditions, after incubation at 37^{O}C in a 5% CO_2 humidified atmosphere for 3hrs, aliquots (99µl) of cell suspension were transferred into pre-warmed (labelled) Eppendorf tubes and treated with 1µl of compounds; to give a final concentration of 100µM compound(s) and 1% DMSO (control). Immediately after treatment, the mixtures were incubated at 37^{O}C in a 5% CO_2 humidified atmosphere for 20min. Change in motility parameter was analysed after 20min using CASA. In brief, 3µl of sample were loaded onto two section of a chamber slide (pre-warmed and maintained at 37^{O}C) and video recording of 16 (randomly selected (minimum of 800 cells)) microscopic fields of sperm sample was made over 2min using negative phase contrast microscopy. Sperm were tracked and analysed using Hamilton-Thorne HTM-IVOS CASA system.

Table I: List of Compounds tested

Tag #	Product Name	Tag #	Product Name
1	Dipyridamole	**23**	A-7 hydrochloride
2	(R)-(-)-Rolipram	**24**	Zaprinast
3	Rolipram	**25**	Cilostazol
4	Vinpocetine	**26**	Ibudilast
5	CGH 2466 dihydrochloride	**27**	Irsogladine maleate
6	W-7 hydrochloride	**28**	Caffeine
7	IBMX	**29**	CP 80633
8	Milrinone	**30**	MMPX (8-MeO-IBMX)
9	BRL 50481	**31**	Siguazodan
10	Mesopram	**32**	EHNA hydrochloride
11	Cilostamide	**33**	Theophylline
12	Pentoxifylline	**34**	YM 976
13	T 0156 hydrochloride	**35**	ICI 63197
14	(S)-(+)-Rolipram	**36**	Etazolate hydrochloride
15	Zardaverine	**37**	Papaverine
16	RS 25344 hydrochloride	**38**	Tofisopam
17	Anagrelide hydrochloride	**39**	Nicardipine
18	MY-5445	**40**	Nimodipine
19	Ro 20-1724	**41**	Tadalafil
20	Sildenafil citrate	**42**	Bay-73-6691
21	Trequinsin hydrochloride	**43**	Bay-60-7550
22	W-9 hydrochloride		

3.3.4 Evaluating the Effect of PDEi(s) on Rate of Sperm Capacitation

The effect of PDEi(s) on rate of sperm capacitation was determined by the ability of capacitated sperm to undergo induced acrosome reaction after treatment with calcium ionophore (10µM) (Liu and Baker, 1998). Semen collected from healthy donor was fractionated by density gradient (Percoll) (see 2.3.1.1 above) and spermatozoa in the 80% interface were washed in 1x concentrated NCM by centrifuging at 500g for 10min. CASA analysis was perfumed and samples were standardised at 20-25x 10^6cells/ml after they were resuspended in CM. Aliquot of 297µl were incubated with 3 µl of compound (100µM), and 1% DMSO (control)) for 2hrs at 37^{o}C in 5% CO_2 humidified atmosphere. To determine capacitation status, aliquot of 50µl were treated with 0.5µl calcium ionophore, A23187 at 10µM final concentration, or 0.5µl of 1% DMSO (control) for 15min at time 0, 1hr and 2hr respectively. Acrosome reacted sperm were identified using fluorescein-labelled *Pisum sativum* agglutinin (PSA). Following incubation with calcium ionophore, 17µl aliquot were pipetted onto clean glass slides and allowed to air dry. To permeabilize the plasma and outer acrosomal membranes, slides were fixed in 100% methanol at room temperature for 20-30min, were gently washed twice with 100µl TBST (Tris-Buffered Saline and Tween 20) and were then labelled with 80µl 1µg/ml FITC-PSA (Sigma L0770) for 30min at room temperature in a dark moist chamber (Cross NL, 1986). Slides were then washed with TBST to remove excess FITC-PSA and mounted with hydromount (National Diagnostics NS-106). AR assays were evaluated with a fluorescence microscope (excitation filter 450–490 nm, emission filter 520 nm). The AR patterns (bright fluorescence: acrosome intact; no fluorescence or only fluorescence of the equatorial segment: acrosome reacted) were evaluated in at least 200 cells per condition. Negative (no stimulation) and positive (A23187 10µM) controls were included in all experiments.

3.3.5 Sperm Penetration Assay

Methyl cellulose (1% concentration) was used to assess sperm functional ability to penetrate into viscous media (Ivic et al., 2002). Sperm cells were previously treated with compound(s) (as described in 3.3.3) and glass tubes filled with 1% methyl cellulose was placed vertically in the sperm solution. Glass tubes (5 cm X 0.8 cm X 2 mm; Vitrocom, NJ, USA) were loaded with methyl cellulose by capillary action (4,000 cP Sigma M-0512) and one extremity was sealed with Cristaseal™ (Hawksley, Sussex, UK). The tubes were incubated with the sperm cells at 37°C for 1h 15min, and then the other end of the tube was sealed after incubation. The number of sperm cells was scored at 1cm using CASA with a 20X objective. The microscope was adjusted manually to obtain the correct focus at each plane to obtain the number of cells migrated. The results are expressed as a penetration index (that is, number of spermatozoa observed with treatment/the number of spermatozoa without treatment).

3.3.6 Assessment of Sperm Damage

The Annexin-V–FLUOS Staining Kit (Roche, Meylan, France) was used to detect phosphatidylserine (PS) translocation from the inner to the outer leaflet of sperm plasma membrane. Annexin V is a phospholipids-binding protein that has high affinity for PS but lacks the ability to pass through an intact sperm membrane (van Heerde et al, 1995). Therefore, any binding between annexin V and PS needs to occur on the outer surface of the sperm plasma membrane, indicating that the membrane integrity has been compromised. Following the instructions of the manufacturer, for each assay, 10^6 cells were washed after 20mins incubation with compounds, and then diluted in 100µl of annexin V buffer. Five microlitres of annexin V–FITC were added to the sample. The tubes were incubated for 15 min at room temperature in the dark; 900 µl of binding buffer and 6µM propidium iodide (PI)

were then added to each tube. Fluorescence microscopy analysis was conducted within 10mins.

3.4 Results

3.4.1 Effects of Vehicle Control (DMSO) on Sperm Motility

All the 43 commercially available compounds used in this study were solubilized in 100% Dimethyl sulfoxide (DMSO) and 1% final concentration of DMSO has been reported to have no detrimental effects on human spermatozoa (de Lamirande and Gagnon, 2002, Wang et al., 2013). To investigate this, capacitated spermatozoa isolated by direct swim-up were exposed to 1%, 0.5% and 0.2% finial concentration of DMSO for 20 min respectively. Direct swim-up method allows isolation of highly motile sperm so the effect(s) of this solvent on total and progressive motility of human spermatozoa will not be compromised by less motile cells. As shown in Figure 3-3A and 3B, the percentages of total and progressively motile cells are not significantly different from control (Capacitating Media: CM). The mean values of other motility parameters such as VCL (p=0.683), VAP (p=0.800), VSL (p=0.713) and hyperactivation (p=0.847) were not affected (data not shown).

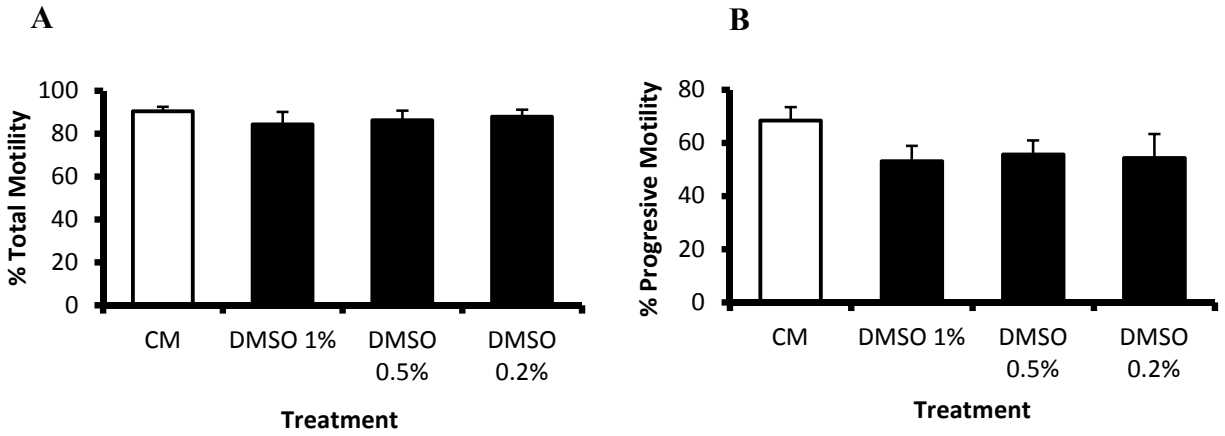


Figure 3-3: Effect(s) of different dimethyl sulfoxide (DMSO) concentrations on human sperm motility: Population of highly motile human spermatozoa was isolated by direct swim up. Cells were incubated under capacitating conditions at 37^{O}C in a 5% CO_2 humidified atmosphere for 3hrs; they were then treated with DMSO (final concentration 1%, 0.5% and 0.2%) or without DMSO (CM: capacitating media) and left to incubate for 20min at 37^{o}C in a 5% CO_2 humidified atmosphere. (A) Percentage total motility (B) Percentage progressive motility. p=0.831 and 0.507 respectively, n=3 (3 different sample from 3 individuals) mean ±SEM (Data not shown: VAP: p=0.800, Hyperactivation: p=0.847 VSL: p=0.713 and VCL: p=0.683). One way-ANOVA

3.4.2 Effects of Compound(s) on Sperm Motility

The initial phase 1 investigation involves the use of poor motility fraction from pooled samples from normozoospermic donors (see Figure 3-2). In order to identify compounds that have significant effect on total motility, cells were incubated under non-capacitating conditions. Response to treatment were categorised as moderate responder (20-60% increase in total motility) and strong responder (> 60% increase total motility) respectively (APPENDIX 1: Figure 1 and 2). Six compounds (#1, #26, #30, #36, #37 and #38) were considered to have strong effect on total motility (Figure 3-4A), therefore they were selected for further analysis.

Further testing (phase 2) on individual donor sample in non-capacitating conditions (n=6: poor motility fraction) showed significant increase in total and progressive motility for all selected compounds (except for #26 total motility) (Figure 3-4B). Evaluation of the effect(s) of these compounds under capacitating conditions (n=7 individual sample; poor motility fraction) (Figure 3-4C) showed similar significant increase in total and progressive motility that was comparable to what was observed when compounds were used in non-capacitating conditions (Figure 3-4B).

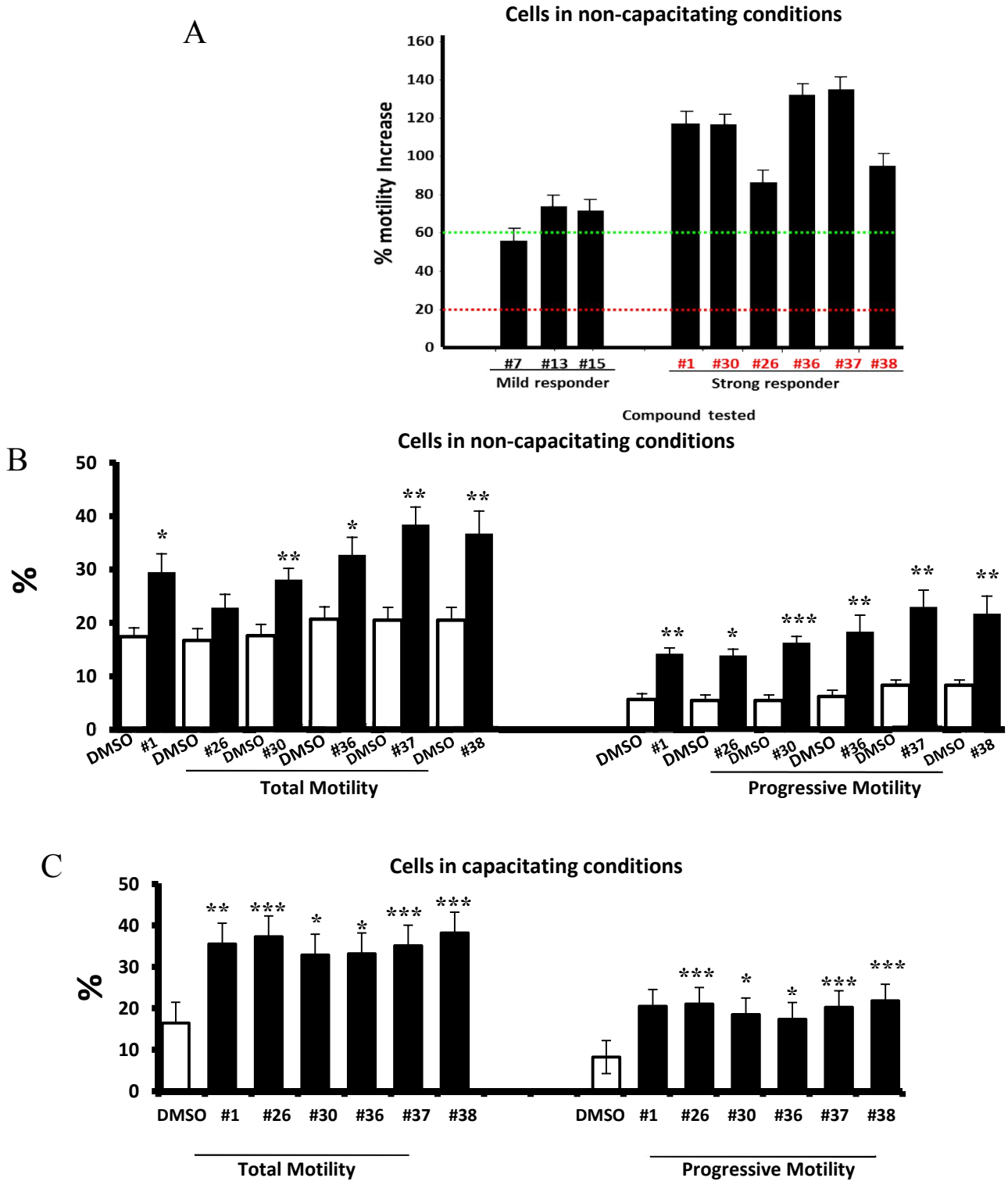


Figure 3-4: Effect(s) of compound(s) on sperm motility: Human spermatozoa were treated for 20min at 37°C with 100µM of Compound. (A) Strong responder (#1, #26, #30, #36, #37 and #38) and Mild responder (#7, #13, #15). 100% increase equal 2 fold increase in motility compare to 1% DMSO (i.e.: DMSO=15%-- treatment 30%). Green line indicates threshold for strong responder while red line is the threshold for background. Red: most promising compounds; n=3 (3 separate analysis on pooled sample) mean ±SEM. (See APPENDIX 1: (Fig 1a-d) for other compound tested). (B) Effect(s) of selected compounds on percentage of motile and progressively motile cell under non capacitating condition. (* p≤ 0.05, ** p≤0.001 and ***p≤0.0001 in comparison to control) n=6 (6 different samples from 6 individuals) mean ±SD. (C) Sperm cells with poor motility from poor fraction were incubated at 37°C in a 5% CO_2 humidified atmosphere for 3hrs; they were then treated with 100µM of compound and left to incubate for 20min at 37°C in a 5% CO_2 humidified atmosphere. (* p≤ 0.05, ** p≤0.001 and ***p≤0.0001 in comparison to control) n=7 (7 different sample from 7 individuals) mean ±SEM.

3.4.3 Preservation of compound(s) effects during prolong co-incubation and removal

Figure 3-4B and C shows the effects of selected compounds on total and progressive motility after a period of 20min incubation with capacitated and non-capacitated cells respectively. When non-capacitated cells were incubated with compounds over a continues period of up to 180 min, a significant increase in percentage total and progressive motility was generally observed when compared with control (Table II). For total motility (TM), cells incubated in compound #37 and #38 were significantly different to control at each time point (Table IIA). Cells incubated in compound #36 were significantly different from control at T20, T60, T90, and T120. Cells incubated in compound #26 and #30 were significantly different from control at T20, T60, T90. Cells incubated in compound #1 only showed a significant difference at T20, T60. The effects of the compounds on total motility were not significantly different from each other, except at T20 where cells incubated in compound #37 were significantly different from #30 (p =0.023). The effects of the compounds are not significantly different over time (e.g. TM: #37 at time T20 is not different from T180). The percentage progressive motility of cells incubated with compounds are significantly different from control at each time point (Table IIB). Cells incubated with compound #38 had a significantly higher progressive motility to those incubated in compound #1 ($p<0.05$) at all time points, compound #30 at 4 time points (T20, T60, T120, and T180) and compound #36 at 1 time point (T90). Cells incubated in compound #37 had significantly higher progressive motility to compound #1 at all time points except T180 and were significantly higher than those incubated in compounds #30 and #36 at T20 and T90 respectively. There is no significant difference between compound #26, #37 and #38 in terms of percentage of cells with progressive motility.

Table II**:** Effects of compound on sperm motility with continuous co-incubation (180min) in non-capacitation conditions

		Total Motility (% ± SEM)[A]							Progressive Motility (% ± SEM)[B]						
		DMSO	#1	#26	#30	#36	#37	#38	DMSO	#1	#26	#30	#36	#37	#38
Time (min)	T20	21 ± 1	32 ± 3*	32 ± 2*	31 ± 1**	33 ± 4*	42 ± 6**	37 ± 11*	8 ± 2	17 ± 3*	19 ± 3*	18 ± 2*	20 ± 3*	30 ± 4**	29 ± 4**
	T40	22 ± 3	32 ± 3	39 ± 6	35 ± 4	35 ± 4	42 ± 7*	37 ± 8*	7 ± 2	18 ± 3*	29 ± 6**	23 ± 3**	23 ± 5*	30 ± 3***	30 ± 4**
	T60	20 ± 2	32 ± 3*	36 ± 4*	32 ± 4*	36 ± 4*	46 ± 13*	43 ± 11*	6 ± 1	18 ± 4*	24 ± 5*	21 ± 4*	23 ± 5*	36 ± 11**	39 ± 9**
	T90	20 ± 2	33 ± 4	34 ± 4*	35 ± 4*	34 ± 3*	46 ± 12**	43 ± 14*	6 ± 2	19 ± 4*	23 ± 4**	23 ± 5*	20 ± 3**	35 ± 7**	39 ± 11**
	T120	20 ± 2	31 ± 4	33 ± 5	30 ± 3	34 ± 4*	40 ± 6**	41 ± 14*	7 ± 1	18 ± 3*	23 ± 6*	17 ± 5	24 ± 4*	29 ± 2***	40 ± 11**
	T180	22 ± 2	32 ± 3	35 ± 4	33 ± 3	36 ± 4	41 ± 6**	38 ± 8*	7 ± 2	20 ± 4*	24 ± 4**	21 ± 3**	24 ± 4*	28 ± 1***	33 ± 5***

n=5 (5 different samples from 5 individuals). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ with respect to control. T20=Incubation for 20min. (A) For total motility, compound #37 and #38 are sig. different to control at each time points. Compound #36 is sig. different from control at time point T20, T60, T90, and T120. Both compound #26 and #30 are sig. different from control at time point T20, T60, T90. Compound #1 is only sig. different at T20, T60. The effects of the compounds on total motility are not sig. different from each other, except at T20 where compound #37 is sig. different from #30 ($p=0.23$). The effects of compounds are not sig. different over time. (B) For progressive motility, all compounds are sig. different from control. Compound #38 is sig. different to compound #1 ($p<0.05$) at all-time point, compound #30 at 4 time points (T20, T60, T120, and T180) and compound #36 at 1 time point (T90). Compound #37 is sig. different to compound #1 at all-time point except T180 and its sig. different to compound #30 and #36 at T20 and T90 respectively.

3.4.4 Persistence of Compound(s) Effect after Drug Removal

In order to mimic clinical procedure in which compounds are washed off, before IVF insemination or intrauterine insemination (IUI), spermatozoa were exposed to compounds for 20min followed by washing off (NCM) and were incubated for up to 300 min. The percentage total and progressive motility were significantly higher in compound treated cells compared to control (Table III).

Specifically for total motility (Table IIIA), cells incubated in compound #1, #26, #36, #37 and #38 showed a significant increase in comparison to control, at each time points except at W20 (compound #38) and W20 and W60 (compound #1). There was no significant difference in cells incubated in compounds before (T20) and after (W0-W300) wash off (i.e. W0-W300). Cells incubated in compound #30 only showed a significant increase in % of motile cells at T20, W40 and W300 respectively.

Specifically for progressive motility, cells incubated in all compound showed a significant increase at T20 (Table IIIB). After washing, cells incubated in compound #36 and #37 showed a significant increase at each time point and compound #26 and #38 are not significantly different from control only at W20. Cells incubated in compound #30, however, do not show any significant difference to control. Cells incubated in compound #1 were only significantly different at W0, W40, and W180-W300.

Table III: Effects of compound on sperm motility after compound was removed after 20min of treatment

		Total Motility (% ± SEM)[A]							Progressive Motility (% ± SEM)[B]						
		DMSO	#1	#26	#30	#36	#37	#38	DMSO	#1	#26	#30	#36	#37	#38
Time (min)	T20	20± 1	35± 0***	38± 2**	36± 2**	37± 1***	42± 2***	40± 2***	8± 1	21± 1***	24± 3**	20± 3**	24± 1***	27± 2***	28± 2***
	W0	18± 1	29± 3*	28± 3*	26± 4	28± 3*	30± 2**	31± 3**	7± 1	18± 3*	16± 3*	14± 3	18± 3*	19± 2**	19± 3**
	W20	21± 3	28± 2	33± 4*	24± 3	31± 3*	36± 5*	33± 5	10± 2	17± 3	20± 4	11± 3	20± 3*	24± 3*	22± 5
	W40	18± 2	29± 2**	30± 4*	30± 4*	27± 3*	33± 4*	36± 2**	7± 1	18± 3*	19± 4*	14± 3	17± 3*	23± 4**	24± 2***
	W60	18± 2	28± 4	32± 4*	25± 3	29± 3*	35± 2**	32± 4*	7± 1	16± 4	19± 3*	13± 3	19± 3**	23± 3**	22± 3**
	W120	18± 2	26± 3*	35± 4**	25± 4	33± 6*	34± 4**	34± 5*	8± 2	15± 2	22± 4*	12± 4	21± 4*	21± 3**	23± 5*
	W180	17± 2	30± 3*	32± 4*	24± 3	29± 3*	34± 3**	33± 4**	7± 2	18± 3*	21± 5*	12± 3	20± 3**	22± 3**	23± 3**
	W240	18± 2	30± 2**	31± 4*	24± 4	29± 4*	34± 3**	31± 4*	9± 1	17± 3*	20± 4*	12± 3	21± 4*	24± 3**	22± 4*
	W300	15± 1	27± 2**	30± 4*	26± 4*	28± 4*	33± 3**	32± 5*	6± 2	15± 3*	19± 4*	13± 4	19± 4*	21± 3**	22± 4*

n=4 (4 different samples from 4 individuals). * p< 0.05, ** p<0.01 and *** p<0.001 with respect to control. (T20=Incubation for 20min, W= Compound washed off/Removed (min)). (A) Compound #1, #26, #36, #37 and #38 significantly increase % motile cells, in comparison to control, at each time points except compound #38 at W20 and compound #1 at W20 and W60. There is no difference between compounds before (T20) and after (W0-W300) compounds were washed off (i.e. W0-W300). Compound #30 only causes sig. increase in % of motile cells at T20, W40 and W300 respectively. (B) At T20, all compounds show sig. increase in % of progressive motility. After washing off the compounds, compound #36 and #37 showed sig. increase in % of progressive motility at each time point and compound #26 and #38 are not sig. different from control only at W20. Compound #30, however, does not show any sig. difference to control after washing off. Compound #1 is only sig. different at W0, W40, and W180-W300 respectively.

3.4.5 Kremer Penetration Test

Using methylcellulose as cervical mucus substitute, the effect of selected compound(s) on functional ability of the cells to migrate into/within viscous environment was tested. There were significantly higher numbers of cells at 1cm when sperm were incubated in the 6 compounds compared to vehicle control (Figure 3-5). Cell incubated in compounds #26 and #38 showed significantly higher response than #1 and #36. Additionally, cell incubated in compounds #38 showed significantly higher response than those incubated in #30, while cells incubated in compound #37 are not significantly different to other compounds (Figure 3-5).

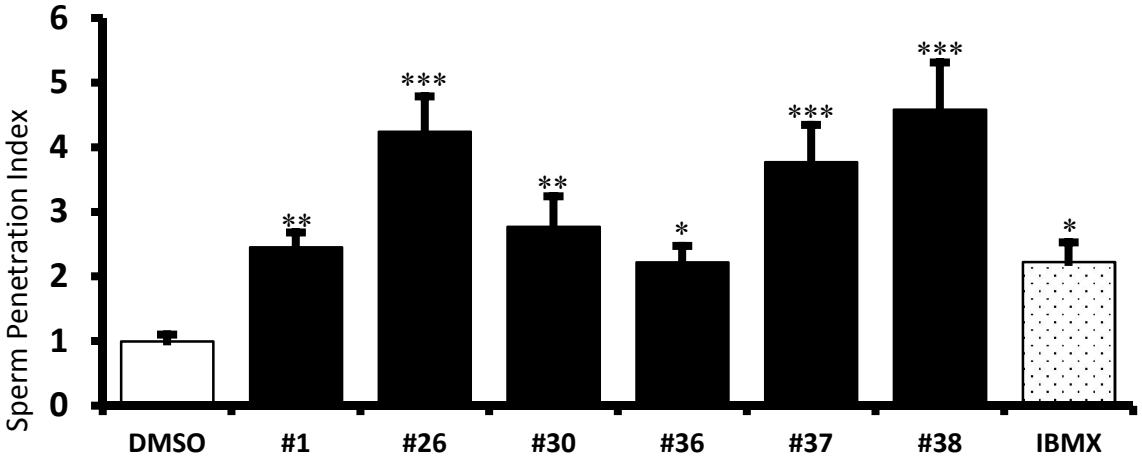


Figure 3-5: Penetration of compound treated and untreated spermatozoa into viscous media: Sperm cells with poor motility were treated with or without 100µM final concentration of Compound(s) (1% DMSO-Control, 500 µM IBMX-Positive control). Treated cells were allowed to penetrate into methylcellulose solution (1cm) for 1hr 15min (Kremer test). (* $p \leq 0.05$, ** $p \leq 0.001$ and ***$p \leq 0.0001$ in comparison to control), n= 8 (8 different samples from 8 individuals) mean ±SEM. Both compound #26 and #38 are sig. different from #1, #36 and IBMX ($p \leq 0.05$). Compound #38 is also sig. different from #30 ($p \leq 0.05$) while #37 is not different from other compounds.

3.4.6 Acrosome Reaction and Externalisation of Phosphatidylserine (PS)

In order to characterise the effects of selected compound(s) on induced acrosome reaction, cells were incubated with compounds and then treated with 10μM (final concentration) calcium ionophore (A23187). Figure 3-6B shows that there was no significant induction of the acrosome reaction after continuous incubation with any of the compounds (p=0.6) compared to vehicle controls (1% DMSO). Also, Figure 3-6A shows that there is no significant difference in externalisation of phosphatidylserine between DMSO treated or compound treated cells.

3.4.7 Effects of Compound(s) on Sperm Movement Characteristics of Patient Samples Incubated in Non-capacitating Media (NCM)

As seen in Table IV, 23 patients were analysed and patients were attending for either routine andrology assessment or IVF/ICSI and a portion of their semen sample was examined following density gradient centrifugation and analysed. It wasn't always possible to examine all 6 compounds due to limitations in number of sperm available. While each patient is an individual there are some general trends. In 3/8 patients with high motility (W.H.O. normal) there was no response (or a negative response) to the compounds (#1, #26, #30 #37 and #38). There was a noticeable number who showed a significant increase in progressive motility to some, but not all compounds. In contrast, in patients with low progressive motility there were a higher proportion of patient samples that responded positively compare to those in the normal category. In patient samples with low total motility and low progressive motility, there was a positive response, either by increase in total motility and/or progressive motility to some, but not all compounds. In this category, only 1 patient showed no response to any compound (RS 760).

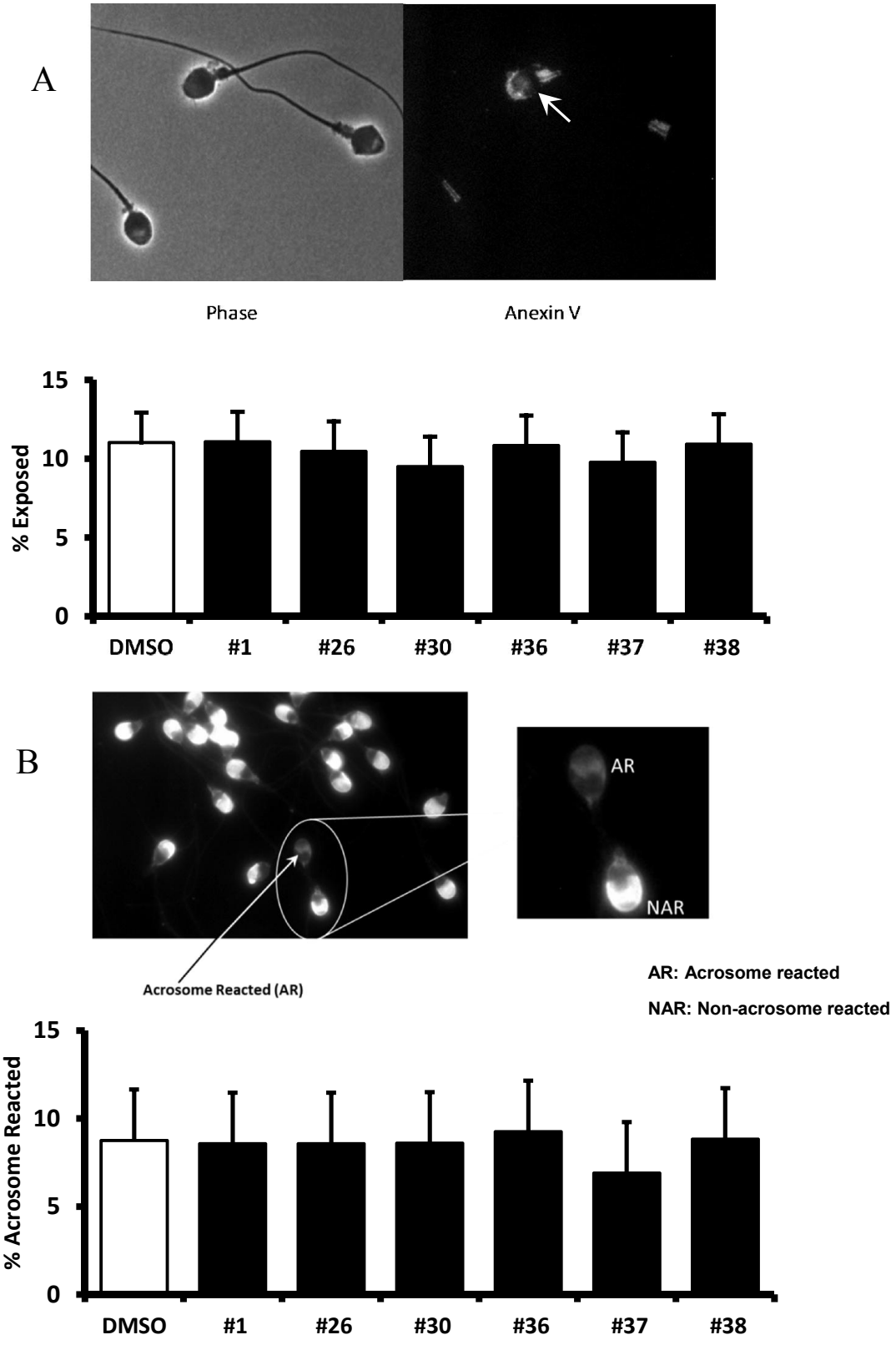


Figure 3-6: Effect(s) of selected compounds on the expression of phosphatidylserine (PS) and percentage of acrosome reacted spermatozoa: Human spermatozoa (sperm fraction with poor motility) were treated with 100µM of Compound. (A) Sperm cells were labeled with fluorescein isothiocyanate-labeled annexin V. Arrow shows cell with no externalization of phosphatidylserine (n=4 (4 sample from 4 individuals); p=0.6. mean ±SEM). (B) Permeabilized spermatozoa were labeled with *pisum sativum lectin* (n=4 (4 samples from 4 individuals); p=0.6 mean ±SD).

Table IV: Summary matrix of patient samples treated with selected compound(s)

~ WHO N = WHO normal, WHO borderline = Borderline motility. Low TM and Low PM = below WHO normal limits for total motility [40%] and progressive motility [32%]. No entry means compound is not tested. Significance means SD do not overlap.

Decreasing total motility

Sample ID	WHO Category	#1		#26		#30		#36		#37		#38	
		TM	PM	TM	PM	TM	PM	TM	PM	TM	PM	TM	PM
721	WHO Normal	↓	↓	×	×	×	×	×	×	×	×	×	×
710	WHO Normal	×	×	↑	×	↑	×	×	↓	↑	×	↑	×
716	WHO Normal	×	↑	×	↑	×	↑	×	↑	×	↑	×	↑
663	WHO Normal	×	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
734	WHO Normal	×	×	×	×	×	×			×	×	×	×
736	WHO Normal	×	×	×	×	×	×			×	×	×	×
774	WHO Normal	×	↑	×	↑	×	↑	×	×	×	↑	×	↑
717	WHO Normal	×	×	×	↑	×	↑	×	↑	×	×	×	×
557	Borderline	↑	×	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
PT01	Borderline			×	↑			↑	↑			×	↑
723	Borderline	×	↑	↑	↑					↑	↑	×	↑
784	Borderline	×	↑	↑	↑	↑	↑	×	↑	↑	↑	×	↑
786	Borderline	↑	↑	↑	↑	↑	↑	×	↑	↑	↑	↑	↑
642	Borderline	↑	↑	↑	↑					↑	↑	↑	↑
653	Borderline	×	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
769	Borderline	×	×	↑	↑	↑	↑	×	↑	×	↑	×	↑
760	Low TM &PM	×	×	×	×	×	×	×	×	×	×	×	×
782	Low TM &PM			↑	↑					↑	↑	×	↑
708	Low TM &PM	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
725	Low TM &PM	×	×	↑	×	×	×	×	×	↑	×	↑	×
709	Low TM &PM			↑	↑								
761	Low TM &PM			↑	↑								
729	Low TM &PM			↑	↑								

↑ Significant Increase × No Change ↓ Significant decrease Drug Was not use in experiment

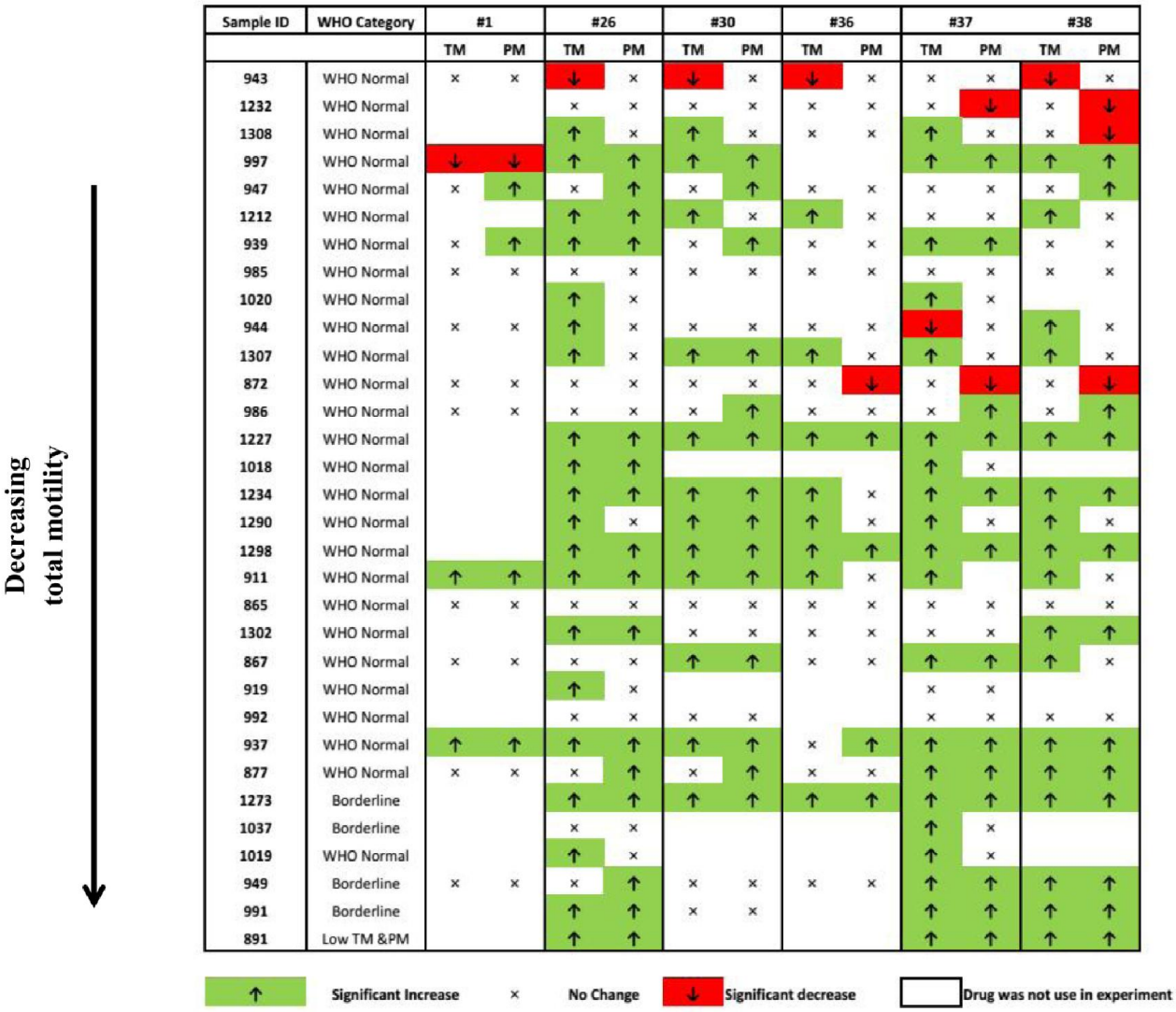
Effect(s) of selected compound on total and progressive motility of patient samples incubated in non-capacitating conditions: Washed samples were categorized into groups by using WHO normal limits for semen sample, that is, total motility [40%] and progressive motility [32%]. Washed sample with ≥ 40% total motility and ≥ 32% progressive motility were categorised as WHO N.

3.4.8 Effects of Compound(s) on Sperm Movement Characteristics of IVF Samples Incubated in IVF Media.

In this category, a portion of prepared patient sample (n=32) that was used for treatment was obtained for research purposes and analysed. Capacitated human spermatozoa from IVF patients were exposed to compounds for 20min (Table V). It wasn't always possible to examine all 6 compounds due to limitations in the number of spermtozoa available. Using W.H.O. 2010 normal limit (40% total motility and 32% progressive motility), 27 out of 32 patients have normal total and progressive motility, 4 were borderline (having either total or progressive motility within normal limit) and 1 patient has lower total and progressive motility (Table V). While each patient is an individual there are some general trends: compounds #37, 38 and 26 were the most effective in increasing the percentage total motile cells (#26 and #37 [20/32] = 63% and #38 [17/27] =63% of patients) followed by #30 [12/26] =46%, #36 [8/22] = 36% and #1 [2/14] =14% respectively Table V. In respect to progressive motility, compound #26 and #30 are the most effective; with ≥50% of samples treated with these compounds having increase in percentage of progressive cells [16/32] =50% and. 54% [14/26] respectively. 41% [13/32] of IVF samples treated with compound #37 and #38 have increase in percentage progressive motility, while 29% [4/14] and 18% [4/22] of samples treated with compound #1 and #36 have increase in percentage of progressively motile cells respectively.

As for samples in non-capacitating media (Table IV), the most significant effects of compounds were in IVF samples with borderline/low motility (Table V).

Table V: Summary matrix of IVF patient samples (in IVF media) treated with selected compound(s)



WHO Normal: WHO normal limits for total motility [40%] and progressive motility [32%], Borderline: Borderline motility, Low TM and Low PM: Both total and progressive motility are below WHO normal limit. No entry means compound is not tested. Significance means SD do not overlap. (TM: Total Motility, PM: Progressive Motility, VCL: Curvilinear Velocity)

3.4.9 Effects of Compounds on Average Swimming Velocity of IVF Samples

In Table V, effect of compounds on total and progressive motility was analysed for each patient sample. Here the effect of compounds on sperm velocity is analysed for each treatment conditions. At a concentration of 100μM, there is a statistically significant increase in VAP (that is, the average velocity of sperm cells over a smoothed cell path) when compared to control (1% DMSO); except for compound #1 treated samples (Figure 3-7A). Although there is no significant difference between other compounds, the most pronounced effects were in #26, #30 and #38 treated cells: the average VAP in DMSO was 60 ±2 μm/s, while that in #26 treated cells was 75 ±2 μm/s, ($p \leq 0.0001$) and 77 ±2 μm/s ($p \leq 0.0001$), 73 ±2 μm/s ($p \leq 0.0001$) for #30 and #38 treated cells respectively.

With regards to VSL (the average velocity on a straight-line between the start and the end point of the track), a statistically significant increase was seen with all compounds except with compound #1 treated samples (Figure 3-7B). Once again, the greatest improvements were achieved with compound #26 (64 ±2 μm/s, $p \leq 0.0001$), #30 (68 ±2 μm/s, $p \leq 0.0001$) and #38 (62 ±1 μm/s, $p \leq 0.0001$): the average VSL in control was (52 ±2 μm/s).

For curvilinear velocity (VCL; the actual velocity along the trajectory), a statistically significant increase was observed for compound #26 (112 ±3 μm/s vs 93 ±3 μm/s), #30 (118 ±3 μm/s vs 93 ±3 μm/s), #36 (111 ±3 μm/s vs 93 ±3 μm/s), #37 (115 ±3 μm/s vs 93 ±3 μm/s), and #38 (121 ±3 μm/s vs 93 ±3 μm/s) (Figure 3-7C). In compound #26 treated samples, 49% of cells had VCL ≥120 μm/s, and a noticeable higher percentage of cells treated with compound #30 (46%), #36 (38%), #37 (41%) and # 38 (50%) also had higher VCL values (≥120 μm/s) in comparison to control (Figure 3-8).

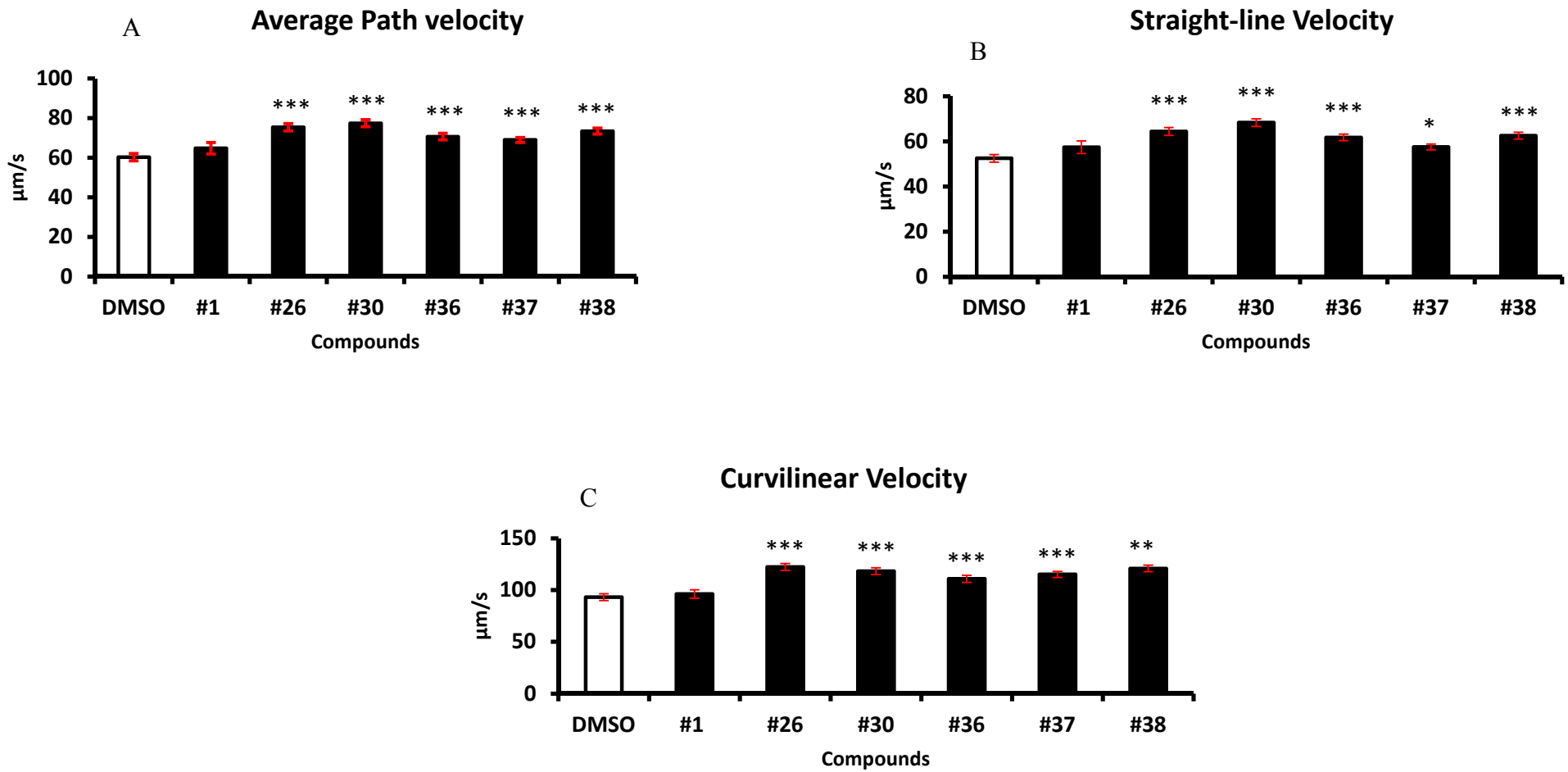


Figure 3-7: Effects of compounds on swimming velocities of spermatozoa from IVF samples. (A) Average path velocity (VAP). (B) Straight-line velocity (VSL). (C) Curvilinear velocity. (DMSO, #26, #37, #38 n=32; #30 n=26; #36 n=22; #1 n=14) mean: ±SEM (* p≤ 0.05, ** p≤0.001 and ***p≤0.0001. One way ANOVA)

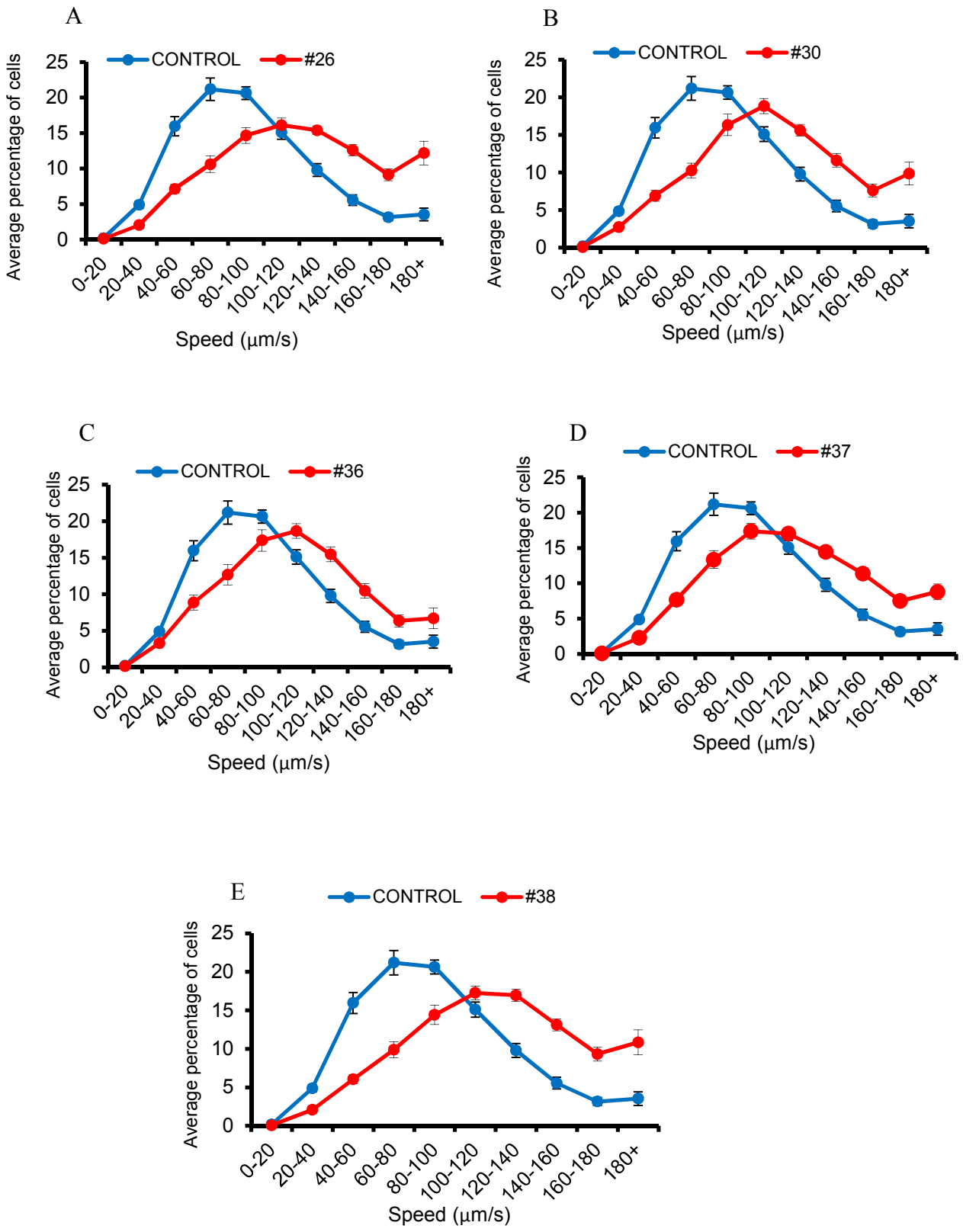


Figure 3-8: The effects of compounds (five compounds only) on cell frequency distribution of VCL following 20min incubation of capacitated spermatozoa from IVF samples. (A) Compound #26 n=32. (B) Compound #30 n=26. (C) Compound #36 n=22. (D) Compound #37 n=32. (E) Compound #38 n=32. Control (1% DMSO n=32), mean ±SEM. On average 230 cells were counted

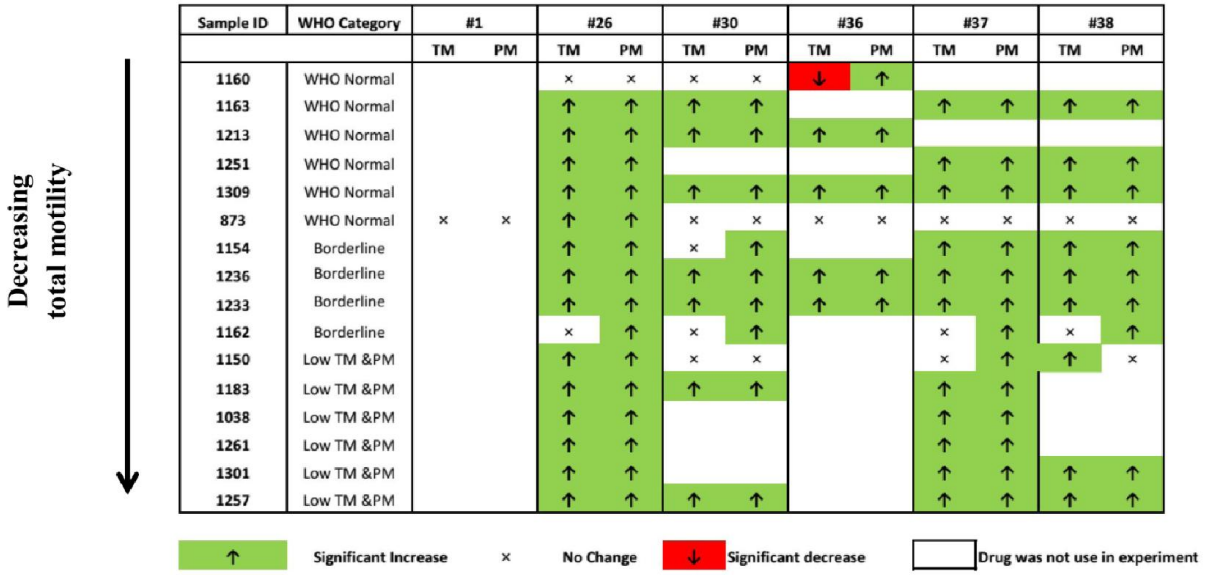
3.4.10 Effects of Compound(s) on Sperm Movement Characteristics of ICSI Samples Incubated in IVF Buffer.

In this category, a very small volume of prepared patient samples were obtained for research purpose; therefore analysis is limited (n=16). Human spermatozoa from ICSI patients were exposed to compounds for 20min (Table VI). It wasn't always possible to examine all compounds due to limitations in number of sperm available.

Using W.H.O. 2010 normal limit (40% total motility and 32% progressive motility), 6 out of 16 patients have normal total and progressive motility, 4 were borderline (having either total or progressive motility within normal limit) and 6 patient has lower total and progressive motility (Table VI). Specifically for total motility, [14/16] =88%, [9/11] =81% and [11/14] =79% of samples incubated with compound #26, #38 and #37 showed a significant increase in total motility respectively. While for compound #30 and #36, [7/12] =58% and [4/6] =67% cases showed increase in total motility (Table VI). In one case, compound #36 causes a decrease in total motility (RS1160). Compound #1 was only tested once in this category because (1) it does not cause statistically significant effect(s) on IVF patient samples; (2) small volumes of patient samples were available for analysis (Table VI).

In respect to progressive motility, compound #26 and #37 are the most effective; with ≥90% of samples treated with these compounds having increase in percentage of progressive cells [15/16] =94% and [13/14] =93% respectively. Great than 80% of ICSI samples treated with compound #36 [5/6] =83% and #38 [9/11] 81% have increase in percentage progressive motility, while 75% [9/12] of samples treated with compound #30 have increase in percentage of progressively motile cells (Table VI).

Table VI: Summary matrix for ICSI patient samples (in IVF buffer) treated with selected compound(s)



WHO Normal: WHO normal limits for total motility [40%] and progressive motility [32%], Borderline: Borderline motility, Low TM and Low PM: Both total and progressive motility are below WHO normal limit. No entry means compound is not tested. Significance means SD do not overlap. (TM: Total Motility, PM: Progressive Motility, VCL: Curvilinear Velocity)

3.4.11 Effects of Compounds on Average Swimming Velocity of ICSI Samples

The effect of compounds on sperm velocity of ICSI samples was analysed for each treatment conditions. At a concentration of 100μM, there is a statistically significant increase in VAP (that is, the average velocity of sperm cells over a smoothed cell path) when compared to control (1% DMSO) (Figure 3-9A). Although there is no significant difference between other compounds, the most pronounced effects was in #26, #37 and #38 treated cells: the average VAP in DMSO was 43 ±3 μm/s, while that in #26 treated cells was 63 ±3 μm/s, ($p \leq 0.0001$) and 59 ±3 μm/s ($p \leq 0.001$), 59 ±4 μm/s ($p \leq 0.0001$) for #37 and #38 treated cells respectively.

With regards to VSL (the average velocity on a straight-line between the start and the end point of the track), a statistically significant increase was seen with all compounds (Figure 3-9B). Once again, the greatest improvements were achieved with compound #26 (54 ±2 μm/s, $p \leq 0.0001$) and #38 (52 ±4 μm/s, $p \leq 0.0001$): the average VSL in control was (33 ±2 μm/s).

For curvilinear velocity (VCL; the actual velocity along the trajectory), a statistically significant increase was observed for compound #26 (97 ±5 μm/s vs 70 ±5 μm/s), #30 (87 ±8 μm/s vs 70 ±5 μm/s), #36 (92 ±7 μm/s vs 70 ±5 μm/s), #37 (94 ±4 μm/s vs 70 ±5 μm/s), and #38 (88 ±6 μm/s vs 70 ±5 μm/s) (Figure 3-9C). In compound #26 treated samples, 28% of cells had VCL ≥120 μm/s, and a higher percentage of cells treated with compound #30 (20%), #36 (26%), #37 (23%) and # 38 (22%) also had higher VCL values (≥120 μm/s) in comparison to control (Figure 3-10).

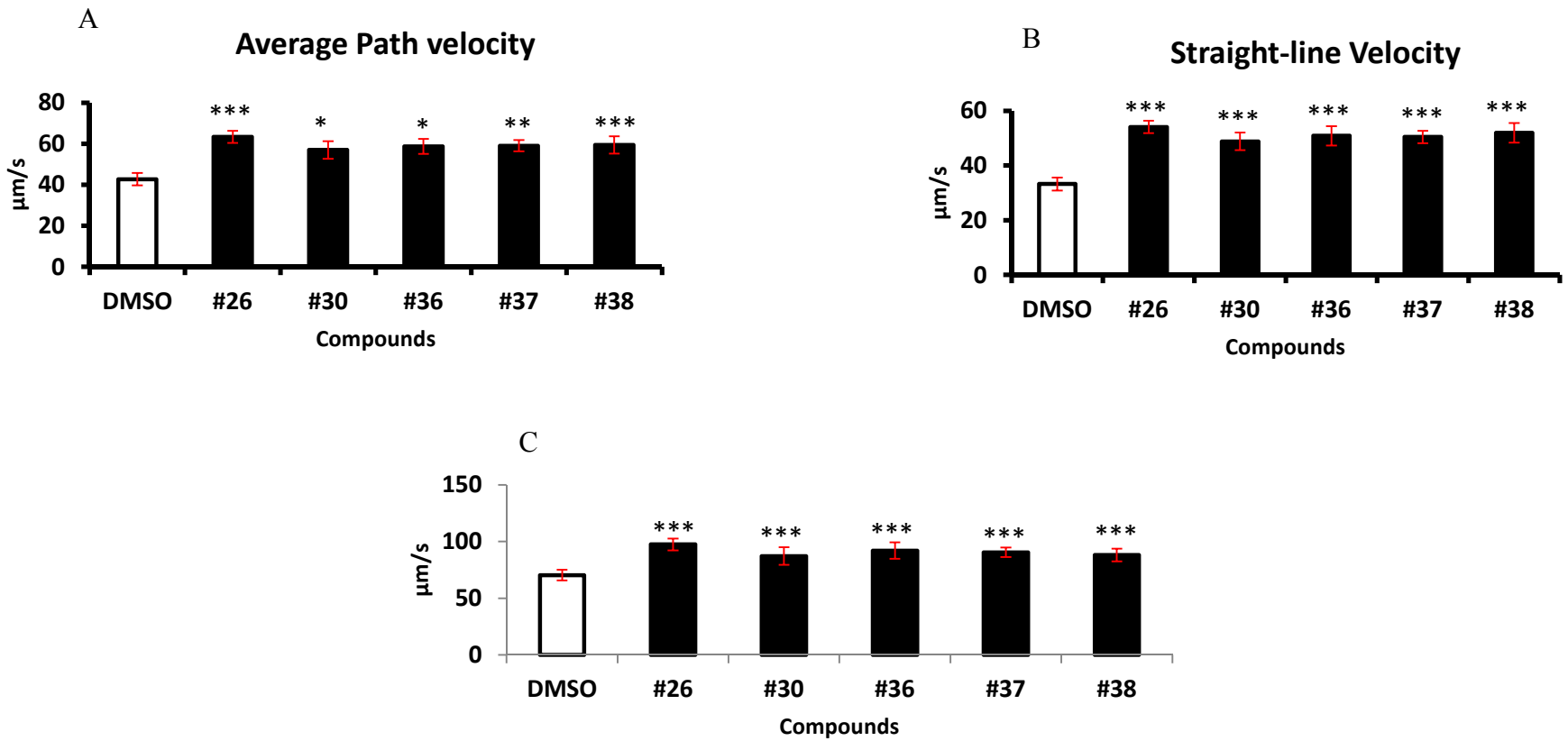


Figure 3-9: Effects of compounds on swimming velocities of spermatozoa from IVF samples. (A) Average path velocity (VAP). (B) Straight-line velocity (VSL). (C) Curvilinear velocity. (DMSO and #26, n=16; #30 n=12; #36 n=6; #37 n=14 and #38 n=11) mean: ±SEM (* $p \leq 0.05$, ** $p \leq 0.001$ and ***$p \leq 0.0001$. One way ANOVA)

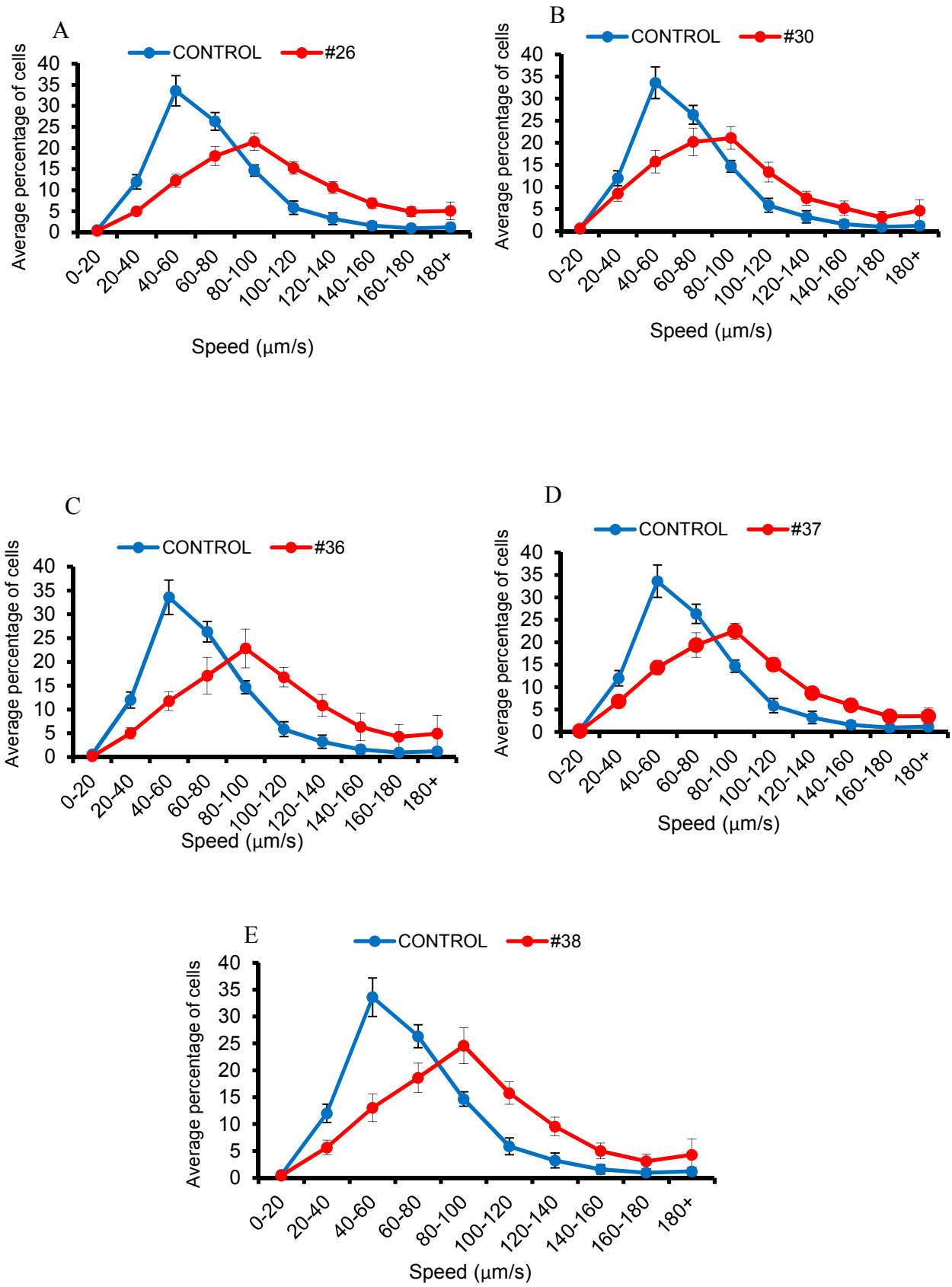


Figure 3-10: The effects of compounds (five) on cell frequency distribution of VCL following 20min incubation of spermatozoa from ICSI samples. (A) Compound #26 n=16. (B) Compound #30 n=12. (C) Compound #36 n=6. (D) Compound #37 n=14. (E) Compound #38 n=11. Control (1% DMSO n=32), mean ±SEM. On average 200 cells were counted.

3.5 Discussion

The aim of this chapter was to use a two phase drug screening approach to identify compounds that have effective stimulation of human sperm motility and to characterise the effects of these compounds on other sperm function. The data presented in this chapter demonstrated that several compounds that have robust and effective stimulation of sperm motility, are non-toxic to the cells, initiate a functional improvement as judged by Kremer testing and importantly have a positive response on a significant proportion of patient samples under clinical conditions have been successfully identified.

In phase 1, 43 commercially available compounds with reported PDEI activity were screened for their effects on sperm motility using CASA. In order to reduce variability and increase the number of cells available for simultaneous examination of multiple compounds (usually 3-5 in each run), pooled samples from 3-4 different donors were utilised. Cells in the 40% fraction (those with poor motility) were used as putative surrogates for patient samples. Previous studies have suggested that these cells have a similar profile, in terms of motility, morphology and DNA status, to men with sperm dysfunction/male infertility (Glenn et al., 2007, O'Connell et al., 2003). The initial screening was performed with non-capacitating media as (1) these are the conditions normally used for IUI (Bjorndahl et al., 2010), (2) there is no bicarbonate stimulation of soluble adenylyl cyclase (sAC) under this condition. Twenty minutes of sperm incubation fits with clinical procedures for preparing cells.

From experiment analysing the effects of the compounds on sperm motility of pooled donor samples, 6 compounds were found to cause ≥60% increase in percentage total motility (Figure 3-4A; APPENDIX 1 Figure 1). When these six compounds were analysed on individual donor samples, in non-capacitating and capacitating media/conditions, a similar

profile of result marked by a significant increase in total and progressive motility was observed (Figure 3-4B and C).

Clinical use of the compounds would involve washing and effective removal prior to use. Table III demonstrates that stimulation of total motility was maintained over time however progressive motility was not consistently affected using compounds #1 and #30. Continual incubation (Table II) suggests that the positive effect on total motility of compounds #1 and #26 and #30 were not maintained throughout incubation. For progressive motility, particular stimulation with compound #38 and #37 was observed (Table II).

The objective of phase 1 was to allow a large number of compounds to be screened relatively quickly to identify potential key targets for further study. Phase 2 consisted of a more detailed assessment based around guidelines for the testing of compounds that can potentially be considered safe for use (Mortimer et al., 2013). Phase 2 involved sperm function testing with a view to the use of the compounds in ART e.g. IUI. For sperm to reach the oocyte it is essential that they can migrate into and within viscous environment found in the female reproductive tract. Modified Kremer testing demonstrated that the stimulation in motility was functional i.e. higher numbers of cells penetrated the viscous media (Figure 3-5).

The purpose of using PDE inhibitors is to improve the quality of sperm from infertile patients, in turn increasing the fertilization potential of these couples. It is therefore important that sperm are both morphologically and physiologically sound to function well in order to fertilize the oocyte. Here, the effects of compounds on A23187 induced acrosome reaction and externalisation of phosphatidylserine (PS) was considered. At 100μM final concentration, the compounds did not appear to have a significant negative effect as there was no significant induction of the acrosome reaction. Additionally, there is no significant PS exposure suggesting that the compounds relatively do not stimulate induction of apoptosis.

This is contrary to what is normally reported. For example, non-specific PDE inhibitor: pentoxifylline (PTX) has been reported to significantly induce acrosome reaction even though it significantly stimulates human sperm motility (Tasdemir et al., 1993, Yovich et al., 1990). One reason for previous association between PDE inhibitors and acrosome reaction might be the concentration of non-selective PDE inhibitors used. For these non-specific inhibitors, motility enhancing effects are only achieved at 1-10mM concentration and these reportedly cause significant premature stimulation of acrosome reaction (Fisch et al., 1998).

The fundamental clinical question is to reproduce what happens in donor samples (40% fraction with poor motility) in patient samples. To address this, a spectrum of diagnostic and treatment samples under non-capacitating and capacitating conditions (Tables IV, V, and VI). In general, in samples with good motility e.g. IVF, there was a minimal effect on total motility but, in some cases, a notable effect on progressive motility. Nevertheless, these compounds significantly enhance the swimming velocity of good motility populations (Figure 3-7) and in most cases ≥ 40% of motile cells had VCL ≥120 μm/s (Figure 3-8).

In samples with lower motility there was a significant effect on both total and progressive motility. These clinical data give some indications as to the possible therapeutic use and effectiveness. Generally there appears to be limited benefit for samples with good motility as expected and consistent with previous data using PTX (Nassar et al., 1999). In cells incubated under non-capacitating conditions 65% and 74% of the samples responded to compound #26 with regards to total and progressive motility. From the limited data available compounds #1 and 30 were less effective. In samples incubated under capacitating conditions (Table V) compounds #37, #38 and #26 were the most effective in increasing the percentage total motile cells (~ 63% of samples) and, as for samples in non-capacitating media, the most significant effects were in samples with borderline/low motility. Only relatively few ICSI samples were examined (Table VI) however, in the overwhelming majority of cases cells

showed an increase in progressive motility and total motility and a considerable higher number of cells had higher VCL.

Chapter 4.

Protein kinase A pathway is critical for basal progressive motility and induction of hyperactivated motility in human sperm

4.1 Introduction**:**

Mature spermatozoa are transcriptionally and translationally silent, which means all sperm functions such as motility, capacitation and acrosome reaction are achieved without activation of genes or production of new proteins (Kiani and Rassoulzadegan, 2013, Braun, 2001, Ward and Zalensky, 1996, Ward and Coffey, 1991). As a result, spermatozoa rely heavily on receptor / ion channel mediated cell signalling and post- translational modification of proteins (such as protein phosphorylation) in order to perform their numerous functions (Florman et al., 1992, Florman et al., 1998, Ren et al., 2001, Quill et al., 2001, Darszon et al., 2005, Lishko et al., 2010, Kirichok and Lishko, 2011, Wassarman, 2002). Detailed studies of sperm capacitation and motility have revealed that modulation of second messenger and kinase activities are central to the signalling pathways that regulate many sperm functions (Tash, 1989, Brokaw, 1987, Salicioni et al., 2007, Leclerc and Goupil, 2002, Breitbart, 2003, Battistone et al., 2013).

Indeed, modulation of intracellular cyclic nucleotide level, particularly cyclic AMP (cAMP), is necessary to activate spermatozoa and is a prerequisite for sperm hyperactivation by mediating flagellar beat frequency (Leclerc et al., 1996a). Soluble adenylyl cyclase (sAC) is the principal enzyme that produces cAMP in mammalian spermatozoa and this atypical enzyme is activated by bicarbonate and calcium (Carlson et al., 2007, Xie et al., 2006). Most evidence has placed cAMP-dependent protein kinase (PKA) as the major downstream effector of cAMP signal in human sperm. Activation of cAMP-dependant protein kinase (PKA) leads to phosphorylation of serine or threonine residues on proteins, leading to phosphorylation of tyrosine residues on other proteins to affect mammalian sperm hyperactivation (Baker et al., 2006, Krapf et al., 2010, Baker et al., 2009)

While compelling experimental evidence has implicated cAMP to be a crucial second messenger that affects human sperm motility, in contrast, there are still controversies regarding the role of cGMP in sperm motility. Using cGMP-dependant phosphodiesterase (PDE) inhibitor (Sildenafil), some studies have demonstrated a stimulatory effect on sperm motility (Lefièvre et al., 2000, Cuadra et al., 2000). At a lower concentration of Sildenafil (0.4 – 40nM), Cuadra *et al*., 2000 reported a significant increase in curvilinear velocity, amplitude of lateral head displacement (ALH), and hyperactivation. Similar results were reported by Lefièvre, et al., 2000, albeit, a higher concentration of Sildenafil was used (10-200µM). Additionally, in this study, increase in intracellular cAMP was also reported suggesting cAMP pathway may perhaps be involved in the motility stimulating effect observed for Sildenafil (Lefièvre et al., 2000).

In contrast, when investigating the effect of Sildenafil (0.26 µM to 1.58µM) on motility, viability, membrane integrity, and functional capacity of human spermatozoa (healthy donors and clinically infertile men), Burger and colleagues found no statistically significant effect of Sildenafil on sperm viability, sperm motility, and sperm progressive motility after incubation of spermatozoa with various doses of Sildenafil (Burger et al., 2000). Similarly, evaluation of the effect of Sildenafil by Andrade and colleagues, on either unwashed or washed spermatozoa of 10 men, demonstrated that 420 µM Sildenafil did not affect sperm motility either in unwashed or washed sperm (Andrade et al., 2000).

Recently, soluble guanylyl cyclase (sGC) has been identified in human sperm using immune-blotting technique (Willipinski-Stapelfeldt et al., 2004) and like in many other cells types, nitric oxide (NO), a free radical, has been suggested to activate this enzyme. NO has been reported to affect sperm motility in a dose-dependent fashion. At low concentration, NO is found to improve or maintain sperm motility probably through the stimulation of cGMP production (Donnelly et al., 1997, Lewis et al., 1996), whereas at higher concentrations of

NO, sperm motility and viability are adversely affected; this is most likely due to free radical ability of NO and direct oxidative damage to the sperm membrane (Rosselli et al., 1995, Weinberg et al., 1995, Balercia et al., 2004).

4.2 Aims and Experimental Design

In Chapter 3, several compounds with reported phosphodiesterase inhibitor activity were identified, and they were found to have effective stimulation of human sperm motility, thus, the aim of this chapter is to identify the causal signalling pathway(s) involved in the motility enhancing effects of these compounds. Since most molecular techniques (such as transfection and protein over-expression) cannot be applied to sperm cells, the use of specific inhibitors of signalling pathways was adopted. Attention was concentrated on PKA, sGC and PKG pathways, which represents key modulator and mediator of cyclic nucleotides in human sperm.

Widely reported chemical inhibitors of PKA (H89: 50μM) (Aitken et al., 1998a, Luconi et al., 2004), sGC (ODQ: 10μM) (Miraglia et al., 2011) and PKG (KT5823: 10μM) (Teves et al., 2009) were used in this study (Figure 4-1). The working hypothesis is that, by inhibiting pathways upstream or downstream of the supposed signalling pathway(s) used by the compounds (PDEi), it is possible to modulate their motility enhancing effects. Under the experimental conditions (Figure 4-2), concentration of chemical inhibitors does not affect sperm viability (as indicated in total and progressive motility studies). In order to minimize possible experimental variations, treatment was performed using the same semen pool. To fully categorise the signalling pathway(s) involved in motility enhancing effects of the compounds identified in Chapter 3, experiments were performed both in capacitating and non-

capacitating conditions. For experiments performed under non-capacitating conditions, sperm cells were pre-incubated (15 min) in the absence or presence of chemical inhibitors (NCM+H89, NCM+ODQ and NCM+KT5823 respectively); in order to ensure inhibition of PKA, sGC and PKG, prior to exposure to compounds (PDEi). Direct exposure of cells to capacitating media could theoretically lead to immediate activation of targeted pathways, thus similar pre-incubation (as above) was done for experiments performed under capacitating conditions; however, pre-incubated cells were then resuspended in capacitating media containing chemical inhibitors, incubated for 3hrs, before exposure to compounds (PDEi).

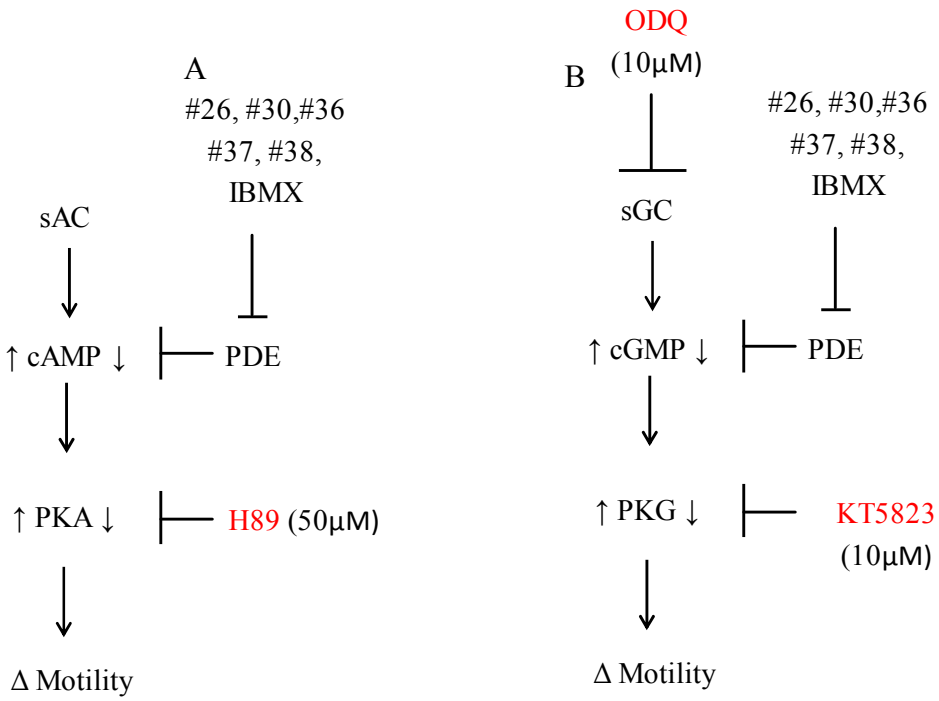


Figure 4-1: Diagram of signalling pathway targeted by chemical inhibitors: (A) PKA activity is inhibited by H89 (as indicated by ⊣) while (B) sGC and PKG activity were inhibited by ODQ and KT5823 respectively. If compounds (PDEi) were working through PKA; inhibiting its activity with H89 will blunt or abolish the motility enhancing effects of compounds. However, if activities of compounds are independent of PKA activation, compounds will still be effective in enhancing sperm motility in the presence of H89. The same principle applies to both sGC and PKG.

4.3 Materials and Methods

4.3.1 Chemicals

Compound(s) were purchased from TOCRIS Bioscience (Bristol, UK), Sigma-Aldrich (Dorset, UK) or Santa Cruz Biotechnology (Heidelberg, Germany) by the Drug Discovery Unit (DDU) at the College of Life Sciences (University of Dundee, UK). Stock solutions of chemical inhibitors, PKA (H89, 100mM), sGC (ODQ 10mM) and PKG (KT5823 10mM), and compounds (PDEi, 10mM) were prepared in DMSO and were kept at 4^{o}C until used. The final concentration of DMSO in the suspensions did not exceed 1% v/v in all treatment conditions.

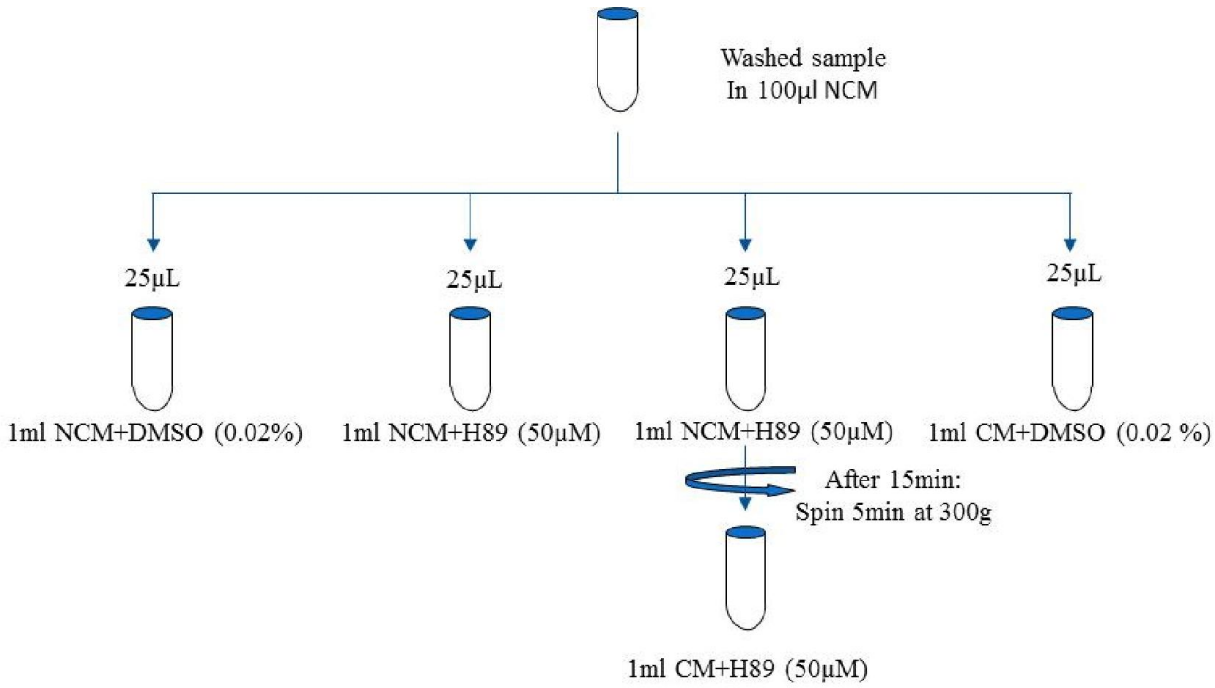


Figure 4-2: Experimental Overview of PKA inhibitor (H89): For each sample, washed sperm cells 100µl was taken and split into four 25µl aliquots. To evaluate the role of PKA, 25µl sample aliquot was resuspended in NCM (with DMSO added as vehicle control) and another in NCM containing H89 (50µM) (NCM+H89); both sample were incubated for 15min at 37^{o}C prior to 20min exposure to compounds (PDEi). In parallel, the other two 25µl aliquots of washed sperm cells was either resuspended in CM (with DMSO added as vehicle control) and incubated for 3hrs under capacitating conditions or in NCM+H89, incubated for 15min at 37^{o}C, pelleted by centrifugation (300g for 5 min) before final re-suspension in CM+H89 and incubation for 3hrs under capacitating conditions. After 3hrs under capacitating conditions, sperm cells were exposed to compounds (PDEi) for 20min. Change in motility was analysed using CASA. Exact experimental conditions were used for ODQ (10µM) and KT5823 (10µM) respectively.

4.3.2 Media and Sperm Preparation

All chemicals were purchased from Sigma-Aldrich. Two different media were used (1) Not supporting sperm capacitation [NCM]: 1.8 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgSO_4.7H_2O$, 116.3 mM NaCl, 1.0 mM NaH_2PO_4, 5.55 mM D-glucose, 2.73 mM sodium pyruvate, 41.75 mM sodium lactate, 25 mM HEPES and 3mg/ml BSA. (2) supporting capacitation [CM]: 1.8 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgSO_4.7H_2O$,116.3 mM NaCl, 1.0 mM NaH_2PO_4, 5.55 mM D-glucose, 2.73 mM sodium pyruvate, 25 mM sodium lactate, 26 mM sodium bicarbonate and 3mg/ml BSA (Alasmari et al., 2013a, Tardif et al., 2014). Semen samples were obtained from healthy donors and population of highly motile sperm cells were isolated using a 40-80% discontinuous density gradient procedure. Briefly, 2 ml of semen was loaded after 30 min of liquefaction at 37˚C on the top of a colloidal silica suspension (Percoll®) made of 80% and 40% layered (2 ml each). The density gradient was centrifuged at 300g for 20 min. Highly motile sperm cell forms a pellet at the bottom of the conical tube within the 80% fraction of the discontinuous density gradient. These cells were recovered and were washed in non-capacitating media (NCM) by centrifugation for 10 min at 500g and the sperm pellet was resuspended in 100µl NCM.

4.3.3 Motility Assessment and Compound(s) Treatment

Washed sperm cells (in 100µl NCM) were split into four treatment conditions. 25µl aliquots (each) was resuspended in 1ml of NCM (with 0.02% DMSO as vehicle control) and in 1ml of NCM containing chemical inhibitors (H89: 50µM, ODQ: 10µM, KT5823:10µM final concentration) and these were incubated under non-capacitating conditions (37^{o}C) for 15 min. In parallel, another 25µl aliquot of washed sperm cells was either resuspended in CM+ (DMSO 0.02%) and incubated for 3hrs under capacitating conditions or resuspended in

NCM+H89, incubated for 15min at 37^{o}C, pelleted by centrifugation (300g for 5 min) before final re-suspension in CM containing chemical inhibitors (H89: 50μM, ODQ: 10μM, KT5823:10μM final concentration) and incubation for 3hrs under capacitating conditions (37^{o}C and 5% CO_2 humidified atmosphere). After respective incubation times (15 min for cells under non-capacitating conditions and 3hrs for cells under capacitating conditions), sperm cells were exposed to compounds (PDEi) for 20min. Change in sperm motility was evaluated using a computer assisted sperm analyser (CASA) (CEROS machine [version 12] Hamilton -Thorne Research, Beverly, MA) attached to an external microscope. Sperm motion characteristics were assessed under a negative phase contrast objective as previously described in Chapter 2 (Alasmari et al., 2013a, Tardif et al., 2014).

4.3.4 STATISTICAL ANALYSIS

All data are presented as means ± SEM. The statistical analysis was carried out using GraphPad Prism 6 (GraphPad Software, California, USA). The data were analysed by two-way ANOVA on paired observations. Where differences were detected, Sidak's and Turkey's multiple comparisons test was used to determine which values differed significantly. Differences were considered significant at a $P < 0.05$. Thus, by using two-way ANOVA, the effects of pre-treatment of cells with or without chemical inhibitors (H89, ODQ and KT5823), in addition to the effects of PDEi inhibitors can be statistically evaluated. Differences between cells with or without chemical inhibitor (H89, ODQ and KT5823) are indicated by asterisk (*), while difference within a treatment group are represented by different letters (a-c) and hashtags (#) respectively.

4.4 Results:

4.4.1 H89 Inhibits Progressive Motility but does not Affect Total Motility under Non-capacitating Conditions

To validate a role for cAMP-PKA pathway in effecting the motility enhancing effects of compound #26, #30, #36, #37 and #38, the effects of PKA antagonist (H89, 50μM) on motile human sperm were examined. Sperm cells were pre-incubated for 15 min, under non-capacitating conditions, with or without 50μM final concentration of H89, before exposure (20 min) to 100μM final concentration of compound(s) (1% DMSO: Vehicle Control and 500μM IBMX: Positive Control) (see Figure 4-2). A two-way factorial ANOVA was conducted on percentage total and progressive motility, with pre-incubation conditions (with or without H89) and treatment conditions (PDEi treatment or DMSO) as factors. In agreement with previous reports (Aitken et al., 1998a), H89 at 50μM has no significant effect on total sperm motility (Figure 4-3A). Thus, when sperm cells are exposed to compounds, total motility did not differ with or without H89 pre-incubation, $p = 0.3635$ (compare white vs black bar chart: Figure 4-3A).

With respect to progressive sperm motility, a significant inhibitory effect was observed (Figure 4-3B). Comparing the effect of pre-incubation of cells with or without H89 showed a significant ($p < .0001$) difference between H89 treated and untreated cells. This result indicate that the percentage of progressively motile sperm was significantly higher for cells treated with compounds but without pre-incubation with H89 than for cells treated with compounds and with initial pre-incubation with H89 (compare white vs black bar chart: Figure 4-3B). A post hoc analysis using Sidak's procedure (α level = 0.05), comparing NCM+(DMSO 0.02%) vs NCM+H89, revealed significant difference in progressive motility for each incubation

conditions (DMSO (1%) and #37: * $p \leq 0.05$); (#36: ** $p \leq 0.01$); (#26, #30, #38 and IBMX: *** $p \leq 0.001$) respectively (compare white vs black bar chart: Figure 4-3B).

Reviewing effects of compounds on the basis of treatment conditions (that is, effect of compound on sperm cells in NCM+DMSO) showed that overall, PDEi treated cells have a significantly (p = .0053) higher % of progressively motile cells than control (1% DMSO) (Figure 4-3B). A post hoc analysis using Tukey's procedure (α level = 0.05), comparing effect of compounds on sperm cells in NCM+DMSO, revealed a significant increase in percentage of progressively motile cells in comparison to control (1% DMSO); (as indicated by letter b #37 $p \leq 0.01$), (as indicated by letters b #26, #30, #36, #38 and IBMX: $p \leq 0.001$) (compare white bar chart: Figure 4-3B). In contrast, for cells pre-incubated in H89 (NCM+H89), there is no significant increase in percentage of progressively motile cells between control and compound(s) treated cells (p >0 .05) (as indicated by hashtag #: compare black bar chart: Figure 4-3B). These results indicated that pre-incubation of sperm cells in H89 (50μM) abolish the motility enhancing effects of the compound(s).

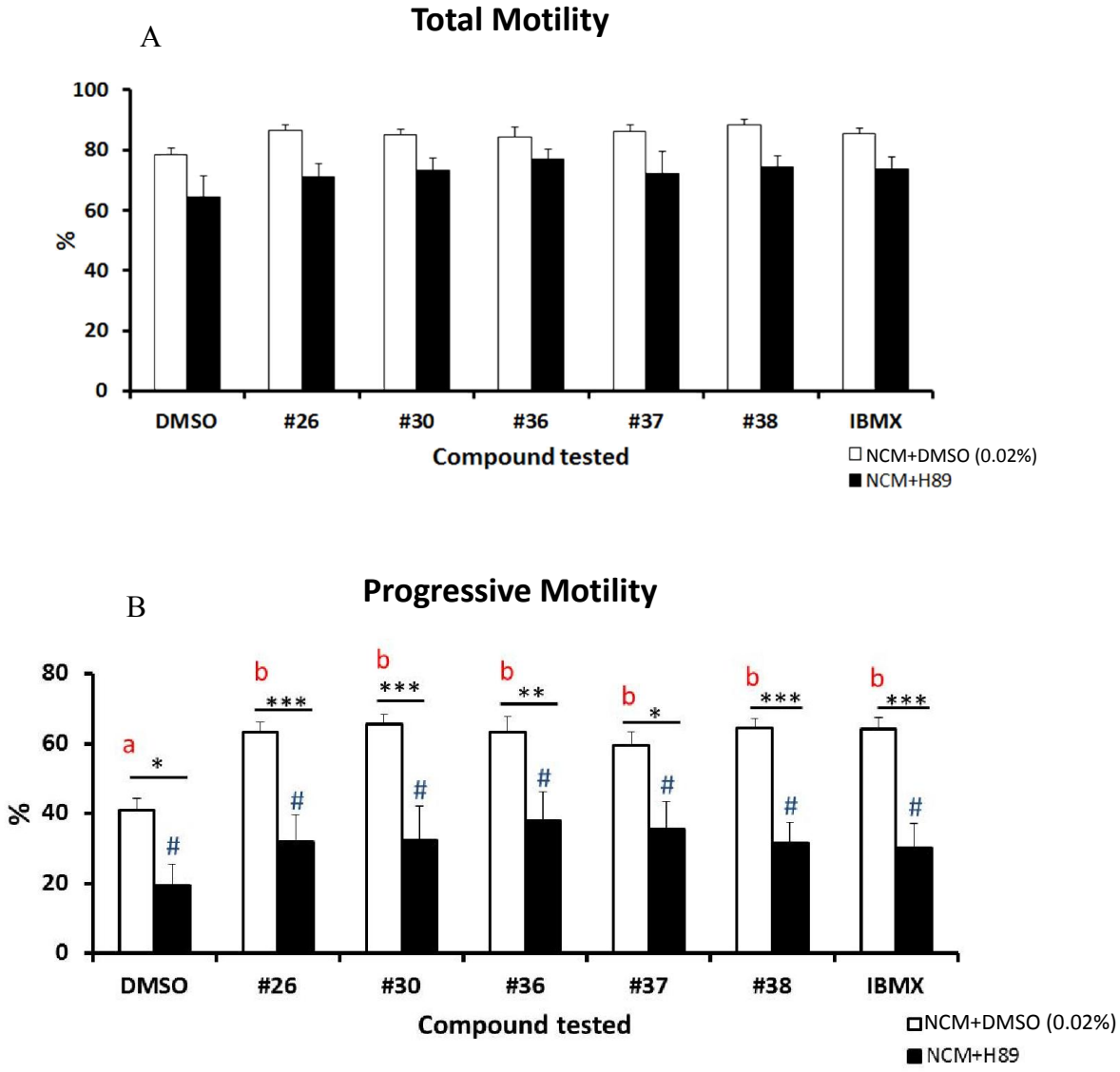


Figure 4-3: Inhibition of PKA with H89 (under non capacitating condition). (A) Total motility is not significantly different between H89 (50µM) pre-treated cells (NCM+H89) and those in NCM+DMSO. (B) Progressive motility is reduced ($P < 0.05$) in H89 (50µM) pre-treated cells in comparison to cells in NCM+DMSO for each compound tested. Addition of compounds did not recover progressive motility. Values shown were measured 20min after addition of compounds to cell pre-incubated (15min) in NCM+H89 and NCM+DMSO respectively. ±SEM n=5-7 (independent experiments): Two Way ANOVA: Differences between cells pre-treated with or without (H89) are indicated by asterisk (*$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$ while difference in a treatment group are represented by different letters (a-b for those in NCM+DMSO: those with different letters differed significantly) and hashtags (# for those in NCM+H89) respectively.

4.4.2 Inhibition of PKA Activity by H89 Affects Sperm Swimming Velocities under Non-capacitating Conditions.

In Figure 4-3 A and B, the effect of compounds on total and progressive motility of cells pre-incubation with or without H89 was analysed. Here the effect of PDEi on sperm velocities is analysed for each treatment conditions. In respect to VAP (that is, the average velocity of sperm cells over a smoothed cell path), there is a statistically significant interaction between compound treatment and the H89 pre-incubation state of the cells. This would suggest that the effect of compounds depended on PKA activity status of the cells (Figure 4-4A). Comparing NCM+DMSO vs NCM+H89 revealed a significant difference in VAP as a result of incubation conditions (#26, #30, #36, #37 #38 and IBMX *** $p \leq 0.001$), with sperm cells in NCM+DMSO having higher VAP than those in NCM+H89 respectively (compare white vs black bar chart: Figure 4-4A).

A simple effects analysis of treatment conditions indicated that overall, the mean VAP for the PDEi treated samples were significantly (****$p<0.0001$) different from control sample in cells pre-treated in NCM+DMSO. A post hoc analysis using Tukey's procedure ($\alpha=0.05$) revealed that the mean VAP of all PDEi treated cells was significantly higher than the mean VAP for control (1% DMSO); (as indicated by letter b #37: $p \leq 0.01$) (as indicated by letter b #26, #30, #36, #38 and IBMX: $p \leq 0.0001$). No significant difference in VAP was found when the PDEi treated cells where compared with each other (compare white bar chart: Figure 4-4A). However, for sperm cells incubated in NCM+H89, there is no significant difference (p >0 .05) in mean VAP between control and PDEi treated cells (as indicated by hashtag #: compare black bar chart: Figure 4-4A).

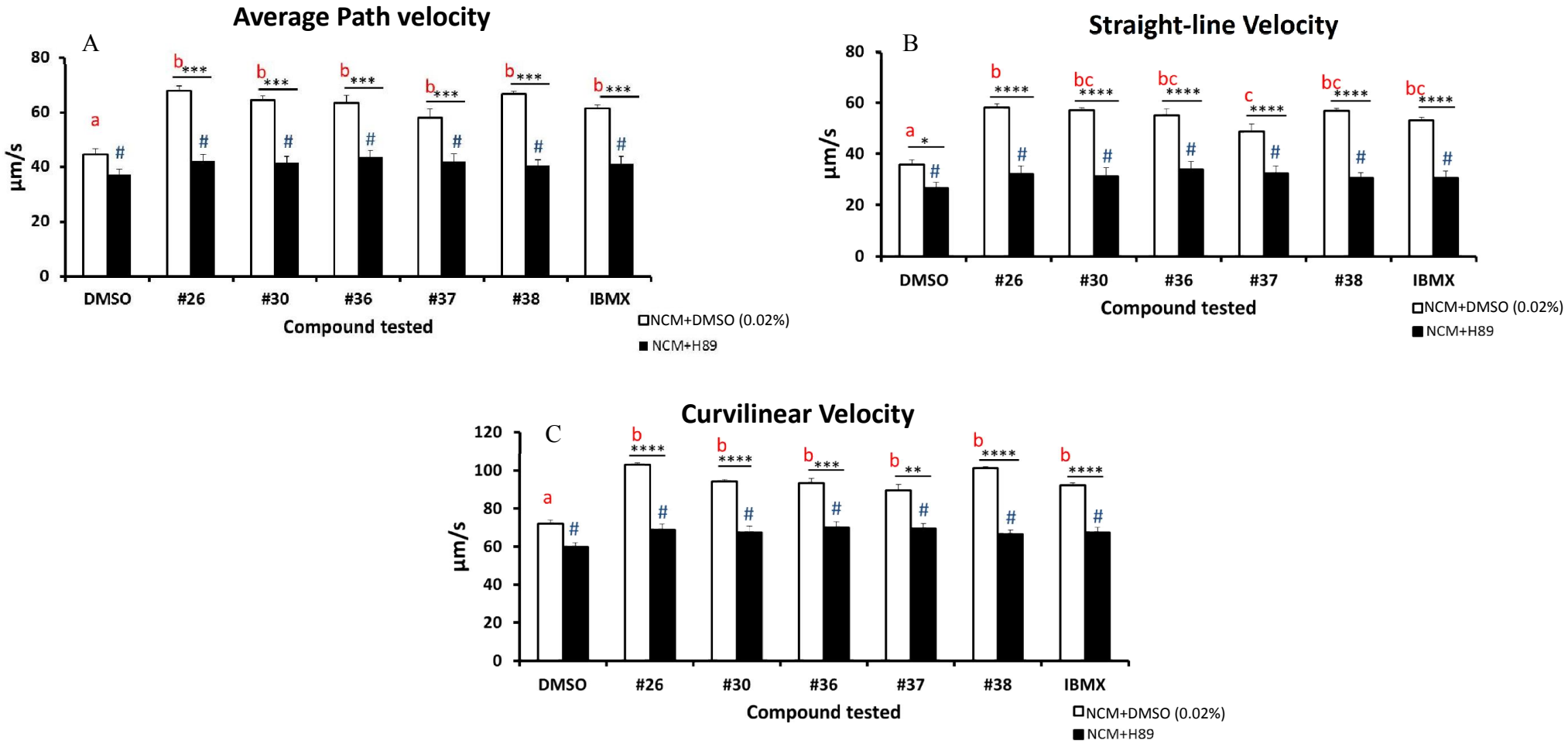


Figure 4-4: Inhibition of PKA with H89 (under non capacitating condition). (A) Average path velocity (B) Straight-line velocity and (C) Curvilinear velocity are reduced ($P < 0.05$) in H89 (50µM) pre-treated cells in comparison to cells in NCM+DMSO. Values shown were measured 20min after addition of compounds to cell pre-treated (15min) in NCM+H89 and NCM+DMSO respectively. ±SEM n=5-7 (independent experiments): Two Way ANOVA: Differences between cells pre-treated with or without (H89) are indicated by asterisk (*$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$). While difference in a treatment group are represented by different letters (a-c for those in NCM+DMSO: those with different letters differed significantly) and hashtags (# for those in NCM+H89) respectively.

With regards to VSL (the average velocity on a straight-line between the start and the end point of the track), there is also a statistically significant interaction between compound treatment and H89 pre-incubation state of the cells (Figure 4-4B). Comparing NCM+DMSO vs NCM+H89 revealed a significant difference in VSL as a result of incubation conditions (#26, #30, #36, #37 #38 and IBMX **** $p \leq 0.0001$), with sperm cells in NCM+DMSO having higher VSL than those in NCM+H89 respectively (compare white vs black bar chart: Figure 4-4B).

Comparing the effects of compounds on the basis of treatment conditions indicated that the mean VSL for the compounds were significantly (****$p<0.0001$) different for sperm cells in NCM+DMSO. A post hoc analysis using Tukey's procedure ($\alpha=0.05$) showed that the mean VSL of all PDEi treated cells was significantly higher than the mean VSL for control (1% DMSO); (as indicated by letter c #37: $p \leq 0.001$) (as indicated by letter b and c #26, #30, #36, #38 and IBMX: $p \leq 0.0001$). When cells treated with PDEi were compared, the average VSL of compound #26 was significantly different from #37 (as indicated by letter b vs c: $p \leq 0.05$); other compounds were not significantly different from each other (compare white bar chart: Figure 4-4B). For sperm cells pre-incubated in NCM+H89, there is no significant difference ($p >0 .05$) in mean VSL between control and compound treated cells (as indicated by hashtag #: compare black bar chart: Figure 4-4B).

For curvilinear velocity (VCL; the actual velocity along the trajectory), sperm cells in NCM+DMSO have higher VCL than those in NCM+H89 (#37: ** $p \leq 0.01$) (#36: *** $p \leq 0.001$) (#26, #30, #38 and IBMX **** $p \leq 0.0001$) respectively (compare white vs black bar chart: Figure 4-4C). Also when effect of PDEi on cells incubated in NCM+DMSO was compared, there is a significant increase in average VCL in comparison to control (as indicated by letter b, #37: $p \leq 0.05$), (as indicated by letter b, #36 and IBMX: $p \leq 0.01$), (as

indicated by letter b, #30: $p \leq 0.001$), (as indicated by letter b, #26 and #38: $p \leq 0.0001$) respectively. No significant difference was found when PDEi treated cells were compared with each other (compare white bar chart: Figure 4-4C). The mean VCL of sperm cells incubated in NCM+H89, are not significantly different ($p > 0.05$) to control (as indicated by hashtag #: compare black bar chart: Figure 4-4C).

4.4.3 Capacitating Conditions Restore H89 Inhibited Progressive Motility

Figure 4-3 and Figure 4-4 imply that compound(s) are effective in enhancing progressive motility and velocities of sperm cells under non-capacitating conditions. However, 15 min pre-incubation of cells with H89 (a PKA inhibitor) abolished the motility enhancing effects of compound(s) under these conditions. Therefore, in this section the effects of H89 on sperm incubated under capacitating conditions is assessed. To achieve this, sperm cells were pre-incubated with inhibitor (H89) in non-capacitating media for 15 min, to ensure PKA inhibition prior to exposure to CM, before resuspending the cells in capacitating media that contains H89 (50µM) (CM+H89) and incubation for 3hrs. After 3hrs incubation, cells from each incubation conditions were exposed to compounds for 20 min (see Figure 4-2).

Similar to what was observed under non-capacitating conditions (Figure 4-3A above), exposure of sperm cells to H89 have no significant ($p > 0.05$) effect on total sperm motility (compare white vs black bar chart: Figure 4-5A). Comparison of treatment conditions (that is, sperm cells in CM+DMSO only or CM+H89 only), also indicates that there is no significant difference in total motility between control and compound treated samples; CM+DMSO: $p = 0.8721$ (compare white bar chart: Figure 4-5A), CM+H89: $p = 0.9981$ (compare black bar chart: Figure 4-5A) respectively. However, contrary to the inhibitory effect of H89 on progressive motility of sperm cells under non- capacitating conditions, H89 has no significant ($p > 0.05$) effect on progressive motility when sperm cells were incubated under capacitating

conditions (compare white vs black bar chart: Figure 4-5B). This result implies that resuspending cells in capacitating media (CM+H89) restores progressive motility inhibited by H89 under non-capacitating conditions. Multiple comparison tests indicates that there is no significant (indicated by letter a: p >0.05) difference in progressive motility when control and PDEi treated samples are compared, however, cells treated with compound #36 have significantly higher (*$p \leq 0.05$) progressively motile cells than cells treated with compound #26 (indicated by letter b vs c: compare white bar chart: Figure 4-5B). For cells incubated in CM+H89, there is no significant difference between control and PDEi treated samples: p =0.8493 (as indicated by hashtag #: compare black bar chart: Figure 4-5B).

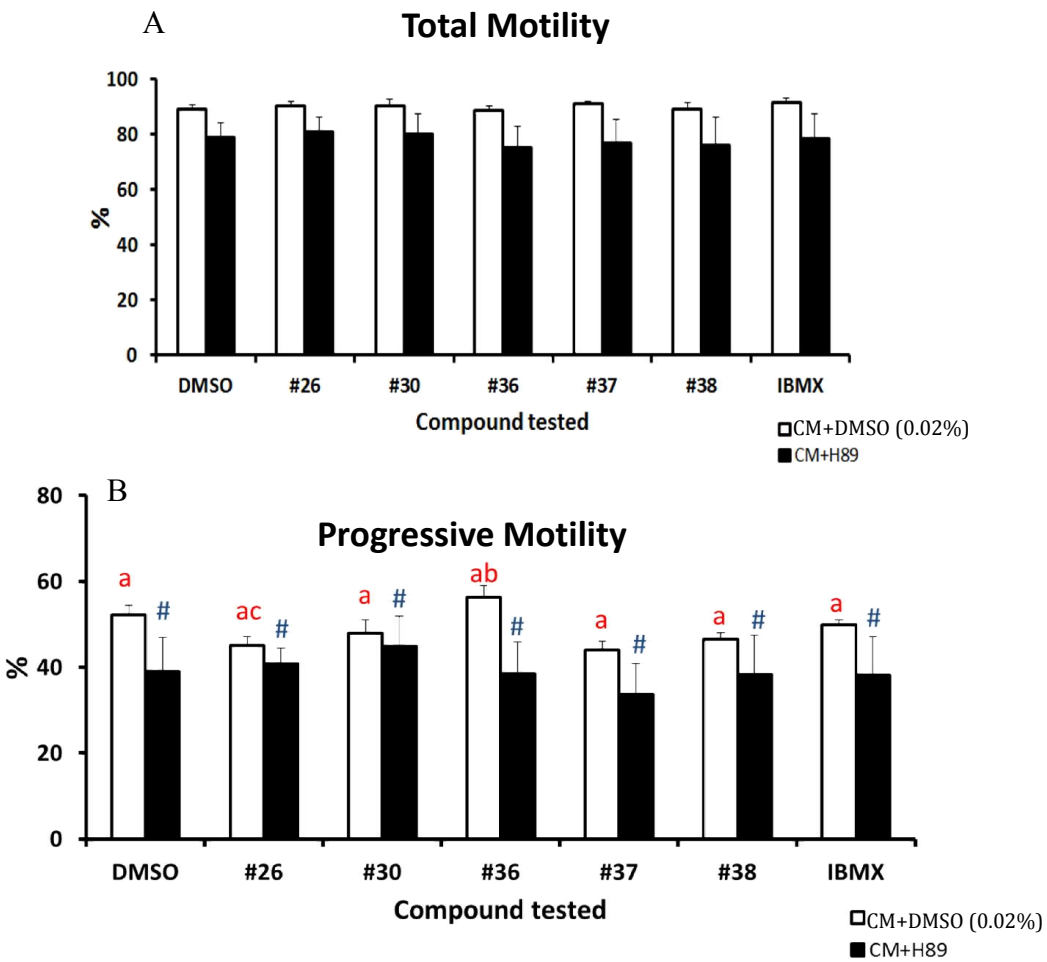


Figure 4-5: Capacitating conditions overcomes H89 mediated PKA inhibition of progressive motility. (A) Total motility and (B) Progressive motility are not significantly (p>0.05) different between H89 (50µM) pre-treated cells (CM+H89) and those in CM+DMSO. Values shown were measured 20min after addition of compounds to cells in CM+H89 and CM+DMSO respectively. ±SEM n=5-7 (independent experiments): Two Way ANOVA: Difference in a treatment group are represented by different letters (a-c for those in CM+DMSO: those with different letters differed significantly) and hashtags (# for those in CM+H89) respectively.

4.4.4 H89 Causes a Reduction in Sperm Swimming Velocities under Capacitating Conditions

Analysis of sperm velocities showed a general trend: sperm cells incubated in CM+DMSO have higher velocities than those incubated in CM+H89 for VAP, VSL and VCL respectively. Particularly for VAP, when comparing CM+DMSO vs CM+H89, there is a statistically significant difference in mean VAP for each treatment condition, (DMSO(1%), #36 and #38: * $p \leq 0.05$), (#26 and #30: ** $p \leq 0.01$), except for #37 and IBMX respectively (compare white vs black bar chart: Figure 4-6A). Multiple comparison tests of cells incubated in CM+DMSO indicates that there is no significant (indicated by letter a: $p > 0.05$) difference in VAP when control and PDEi treated samples are compared, however, cells treated with compound #30 are significantly (*$p \leq 0.05$) different to those treated with IBMX (indicated by letter b vs c: compare white bar chart: Figure 4-6A). For cells incubated in CM+H89, there is no significant difference between control and compound treated samples: $p = 0.9437$ (as indicated by hashtag #: compare black bar chart: Figure 4-6A).

With regards to VSL, comparing CM+DMSO vs CM+H89, there is a significant difference in VSL as a result of incubation conditions, (DMSO(1%), #26, #37 and #38* $p \leq 0.05$), (#30 and #36 ** $p \leq 0.01$), except IBMX (compare white vs black bar chart: Figure 4-6B). The effects of compounds on the basis of treatment conditions indicated that, for cells in CM+DMSO, there is no significant (indicated by letter a: $p > 0.05$) difference in VSL when control and PDEi treated samples are compared, however, cells treated with #30 are significantly (*$p \leq 0.05$) different to those treated with IBMX (indicated by letter b vs c: compare white bar chart: Figure 4-6B). For cells incubated in CM+H89, there is no significant difference between control and PDEi treated samples: $p = 0.9676$ (as indicated by hashtag #: compare black bar chart: Figure 4-6B).

For curvilinear velocity, sperm cells in CM+DMSO have higher VCL than those in CM+H89 (DMSO, #36, #37 and IBMX: * $p \leq 0.05$), (#26 and #38: ** $p \leq 0.01$), (#30: *** $p \leq 0.001$) respectively (compare white vs black bar chart: Figure 4-6C). However, when the effect of PDEi on cells incubated in CM+DMSO was compared with control samples, there is no significant (p=0.0787) increase in mean VCL (indicated by letter a: compare white bar chart: Figure 4-6C). The mean VCL of sperm cells incubated in CM+H89, are also not significantly different (p=0.8852) to control (as indicated by hashtag #: compare black bar chart: Figure 4-6C).

4.4.5 Inhibition of PKA Activity by H89 Leads to Reduction in Percentage of Sperm Cells with Hyperactivated Motility under Capacitating Conditions

The effect of compound(s) on hyperactivated motility of cells incubated with or without H89 was examined under capacitating conditions. Figure 4-7 shows that incubation of cells in CM+H89 causes a significant decrease in percentage of hyperactivated cells; with cells incubated in CM+DMSO having higher percentage of hyperactivated cells for each treatment conditions (DMSO(1%) and IBMX: * $p \leq 0.05$), (#36 and #37: ** $p \leq 0.01$), (#26, #30 and #38: *** $p \leq 0.001$) respectively. Multiple comparison tests of cells incubated in CM+DMSO indicates that there is no significant (p=0.0758) difference in percentage of hyperactivated cells when control and PDEi treated samples are compared (indicated by letter a: compare white bar chart: Figure 4-7). For cells incubated in CM+H89, there is no significant difference between control and compound treated samples: p =0.8373 (as indicated by hashtag #: compare black bar chart: Figure 4-7).

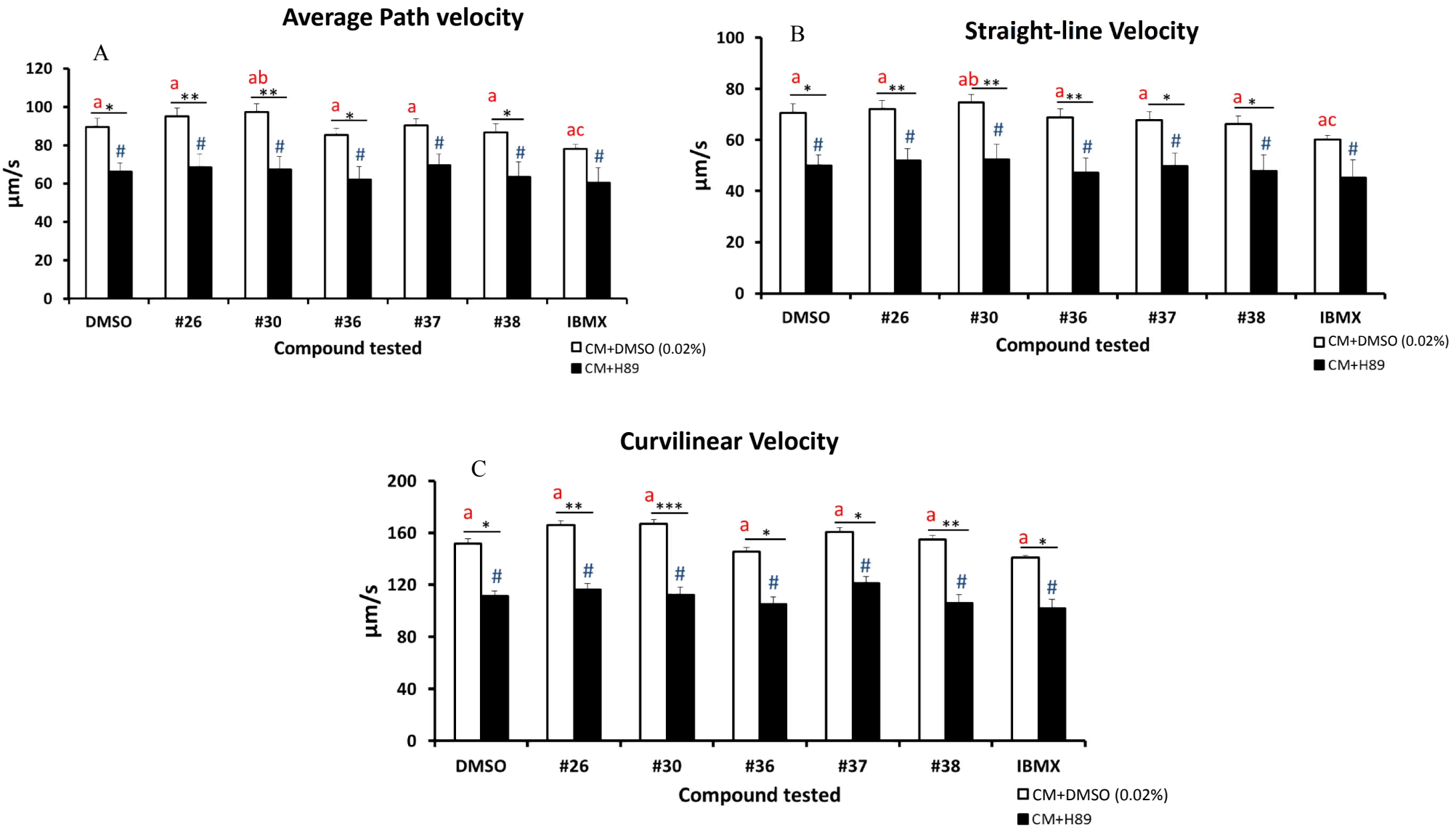


Figure 4-6: Inhibition of PKA with H89 (under capacitating condition). (A) Average path velocity (B) Straight-line velocity and (C) Curvilinear velocity are reduced ($P < 0.05$) in H89 (50µM) pre-treated cells in comparison to cells in CM+DMSO. Values shown were measured 20min after addition of compounds to cell CM+H89 and CM+DMSO respectively. ±SEM n=5-7 (independent experiments): Two Way ANOVA: Differences between cells pre-treated with or without (H89) are indicated by asterisk (*$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$), while difference in a treatment group are represented by different letters (a-c for those in CM+DMSO: those with different letters differed significantly) and hashtags (# for those in CM+H89) respectively.

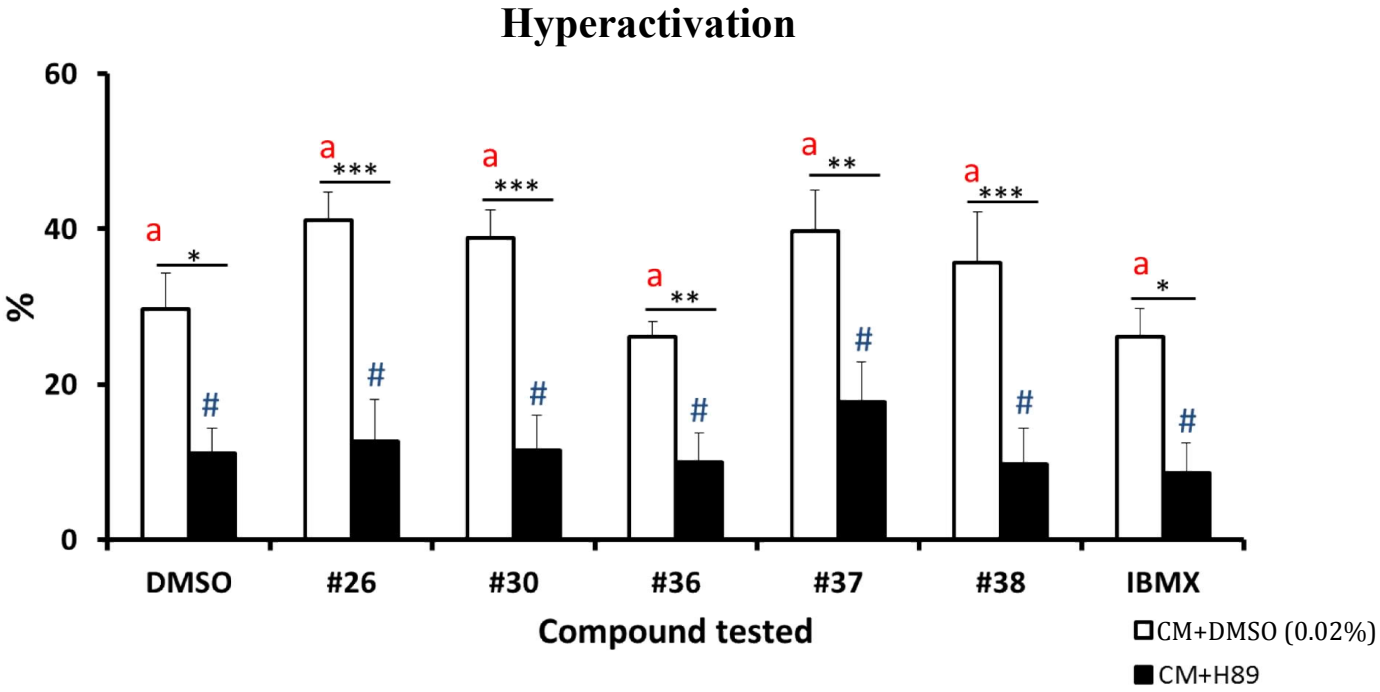


Figure 4-7: Inhibition of PKA with H89 (under capacitating condition) results in decrease in hyperactivation. Percentage of hyperactivated cells is reduced ($P < 0.05$) in H89 (50µM) pre-treated cells (CM+H89) in comparison to cells in CM+DMSO for each compound tested. Values shown were measured 20min after addition of compounds to cells in CM+H89 and CM+DMSO respectively. ±SEM n=5-7 (independent experiments): Two Way ANOVA: Differences between cells pre-treated with or without (H89) are indicated by asterisk (*$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$), while difference in a treatment group are represented by different letters (those with different letters differed significantly) and hashtags (# for those in CM+H89) respectively.

4.4.6 Inhibition of sGC does not Influence Total and Progressive Motility of Sperm Incubated under Non-capacitating Conditions

To establish a role for soluble guanylyl cyclase (sGC) pathway in effecting the motility enhancing effects of compound #26, #30, #36, #37 and #38, production of cGMP was modulated by using ODQ (1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one) (10µM) to inhibit the activity of sGC (Figure 4-8).

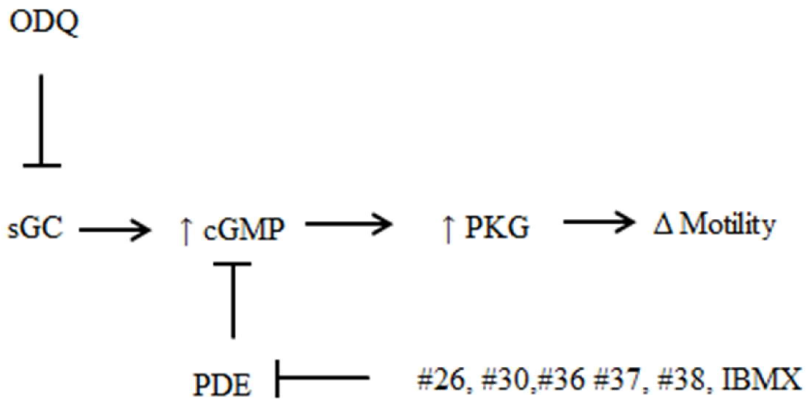


Figure 4-8: Modulation of sGC activity by ODQ (1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one) (10µM) to examine the role of cGMP production to influence human sperm motility.

Sperm cells were pre-incubated for 15 min, under non-capacitating conditions and with or without 10µM final concentration of ODQ, before exposure (20 min) to 100µM final concentration of compound(s) (1% DMSO: Vehicle Control and 500µM IBMX: Positive Control). A two-way factorial ANOVA was conducted on percentage total and progressive motility, with pre-incubation conditions (with or without ODQ) and treatment conditions (PDEi treatment or 1%DMSO) as factors. Figure 4-9A showed that there is no significant difference between total sperm motility when cells incubated in NCM+DMSO and NCM+ODQ were compared (compare white vs black bar chart: Figure 4-9A). This result

indicates that pre-incubation of cells in ODQ (10μM), before exposure to compounds, does not affect total motility of sperm cells. Analysis of progressive sperm motility also showed that there is no significant difference when cells in NCM+DMSO were compared with those in NCM+ODQ (compare white vs black bar chart: Figure 4-9B). However, when treatment conditions (that is, effect of PDEi on sperm cells pre-incubated in NCM+DMSO or in NCM+ODQ) were considered, there was a significant increase in percentage of cells with progressive motility in comparison with respective controls. For cells in NCM+DMSO, post hoc analysis using Tukey's procedure (α level = 0.05), revealed a significant increase in percentage of progressively motile cells in comparison to control (1% DMSO); (as indicated by letter b: #36: $p \leq 0.001$), (as indicated by letter b: #26, #30, #37, #38 and IBMX: $p \leq 0.01$) (compare white bar chart: Figure 4-9B). Similar pattern of significant increase in percentage of progressively motile cell over control was observed for cells in NCM+ODQ, (as indicated by double hashtag (##): #26, #36 and #36: $p \leq 0.0001$), (as indicated by double hashtag (##): #37, #38 and IBMX *** $p \leq 0.001$) (compare black bar chart: Figure 4-9B). These results clearly suggest that pre-incubation of sperm cells in ODQ (10μM), which inhibits sGC activity, does not affect the motility enhancing effects of the compound(s).

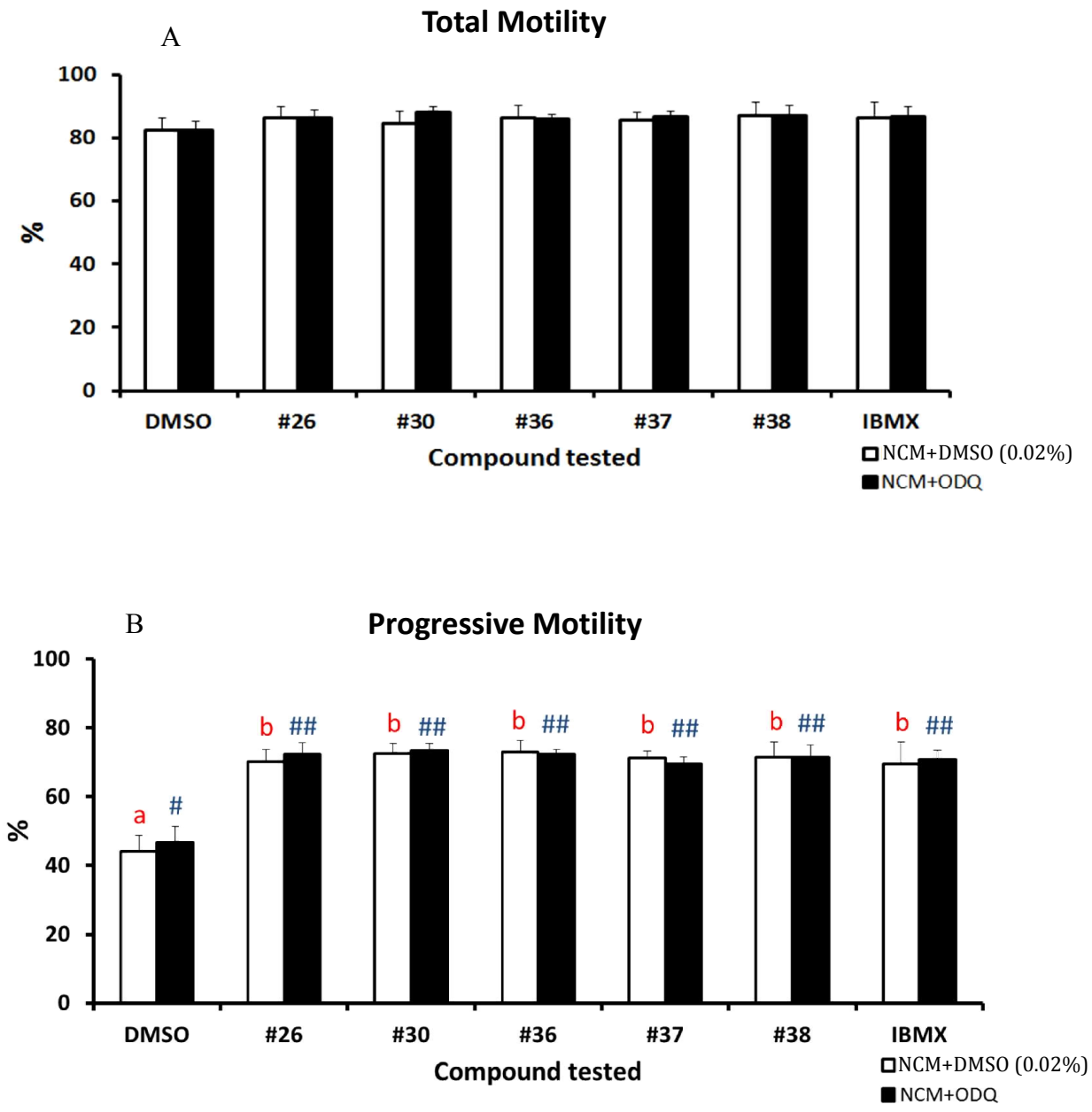


Figure 4-9: Inhibition of sGC with ODQ (under non capacitating condition) does not affect total or progressive motility. (A) Total motility and (B) Progressive motility are not significantly different between ODQ (10µM) pre-treated cells and those in NCM+DMSO. Values shown were measured 20min after addition of compounds to cell pre-treated (15min) in NCM+ODQ and NCM+DMSO respectively. ±SEM n=4 (independent experiments): Two Way ANOVA: Difference in a treatment group are represented by different letters (a-b for those in NCM+DMSO: those with different letters differed significantly) and hashtags (# for those in NCM+ODQ: those with double hashtag differed significantly from those with single hashtag.) respectively.

4.4.7 PDEi Stimulates Sperm Swimming Velocities in the Presence of Chemical Inhibitor of sGC under Non-capacitating Conditions.

Here the effect of PDEi on sperm velocities is evaluated. When velocities of cells incubated in NCM+DMSO were compared with those in NCM+ODQ, there was no significant difference in average velocities for all the velocities analysed: VAP VSL and VCL respectively (compare white vs black bar chart: Figure 4-10).

When treatment conditions (that is, effect of PDEi on sperm cells in NCM+DMSO or in NCM+ODQ) were considered, there was a significant increase in average VAP, VSL and VCL in comparison with respective controls. Particularly for cells incubated in NCM+DMSO, multiple comparison tests indicate that there is significant (*p <0.05) difference in average velocities: VAP - (as indicated by letter b) (#26 and #38: $p \leq 0.001$), (#30, #36 and IBMX: $p \leq 0.01$) (#37 * $p \leq 0.05$) (compare white bar chart: Figure 4-10A), VSL - (as indicated by letter b) (#26 and #36: $p \leq 0.0001$), (#30, #38 and IBMX: $p \leq 0.001$) (#37: $p \leq 0.001$) (compare white bar chart Figure 4-10B), VCL - (as indicated by letter b) (#26 and #38: $p \leq 0.01$) (#30, #36, #37 and IBMX: $p \leq 0.05$) (compare white bar chart Figure 4-10C) when control (DMSO) and PDEi treated samples are compared.

For cells incubated in NCM+ODQ, similar pattern of significant increase in average velocities: VAP - (as indicated by double hashtag ##) (#26, #36 and #38: $p \leq 0.001$), (#30, #37 and IBMX: $p \leq 0.01$) (compare black bar chart: Figure 4-10A), VSL - (as indicated by double hashtag ##) (#26, #36 and #38: $p \leq 0.0001$), (#30 and IBMX: $p \leq 0.001$) (#37: $p \leq 0.001$) (compare black bar chart: Figure 4-10B), VCL - (as indicated by double hashtag ##) (#26 and #38: $p \leq 0.001$)) (#36 and IBMX : $p \leq 0.01$) (#30 and #37: $p \leq 0.05$) (compare white bar chart: Figure 4-10C), over control sample were observed for cells incubated in NCM+ODQ.

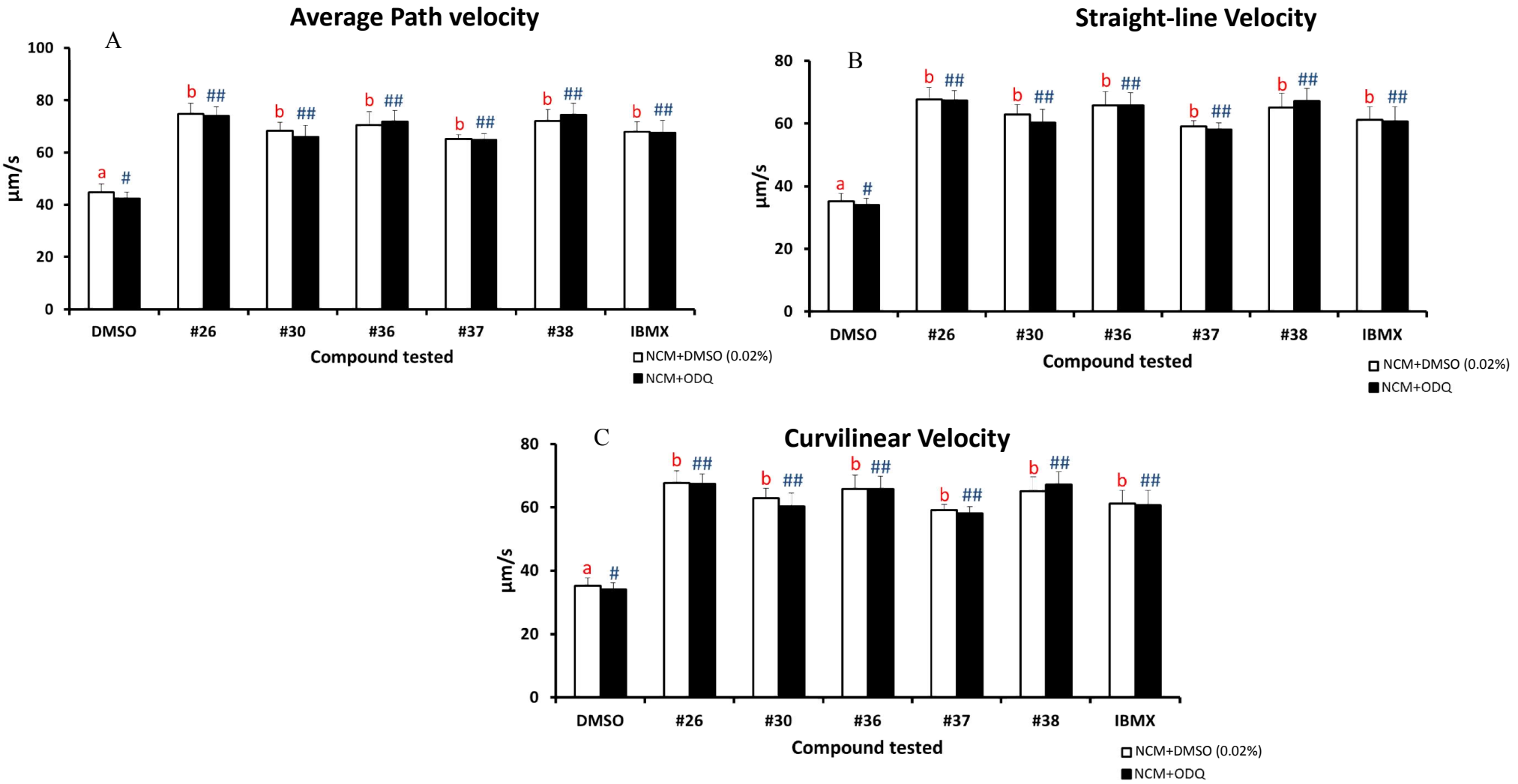


Figure 4-10: Inhibition of sGC with ODQ (under non capacitating condition) does not affect sperm velocity parameters. (A) Average path velocity (B) Straight-line velocity and (C) Curvilinear velocity are not significantly different ($p > 0.05$) in ODQ (10µM) pre-treated cells in comparison to cells in NCM+DMSO. Values shown were measured 20min after addition of compounds to cell pre-treated (15min) in NCM+ODQ and NCM+DMSO respectively. ±SEM n=4 (independent experiments): Two Way ANOVA: Difference in a treatment group are represented by different letters (a-b for those in NCM+DMSO: those with different letters differed significantly) and hashtags (# for those in NCM+ODQ: those with double hashtag differed significantly from those with single hashtag.) respectively.

4.4.8 Inhibition of sGC Activity by ODQ does not Affect Motility of Sperm Cells Incubated under Capacitating Conditions

Result shown in Figure 4-9 and Figure 4-10 unequivocally show that under non-capacitating conditions, pre-incubation of cells in ODQ prior to exposure to compounds does not affect the motility enhancing effects of compounds. Under this condition, exposure of cells to PDEi significantly enhances progressive motility and sperm velocities; there is no significance difference between cells incubated in NCM+DMSO vs NCM+ODQ.

In this section, the effects of ODQ on sperm incubated under capacitating conditions were assessed. Sperm cells were pre-incubated with inhibitor (ODQ) in non-capacitating media for 15 min prior to resuspending the cells in capacitating media that contains ODQ (10μM) (CM+ODQ) and incubation for 3hrs. Alternatively, sperm cells were resuspended in capacitating media that contains 0.02% DMSO (CM+DMSO) and incubated for 3hrs. After 3hrs incubation, cells from each incubation conditions were exposed to compounds for 20 min.

Figure 4-11A and B imply that exposure of sperm cells to ODQ under capacitating condition has no significant (p >0 .05) effect on total and progressive sperm motility (compare white vs black bar chart: Figure 4-11A and B). Comparison of treatment conditions (that is, effect of PDEi on sperm cells pre-incubated in CM+DMSO or CM+ODQ), also indicates that there is no significant difference between control and compound treated samples- total motility CM+DMSO: p = 0.7437 (compare white bar chart: Figure 4-11A), CM+ODQ: p =0.9901 (compare black bar chart: Figure 4-11A); progressive motility CM+DMSO: p = 0.0657 (compare white bar chart: Figure 4-11B), CM+ODQ: p =0.2485 (compare black bar chart: Figure 4-11B) respectively.

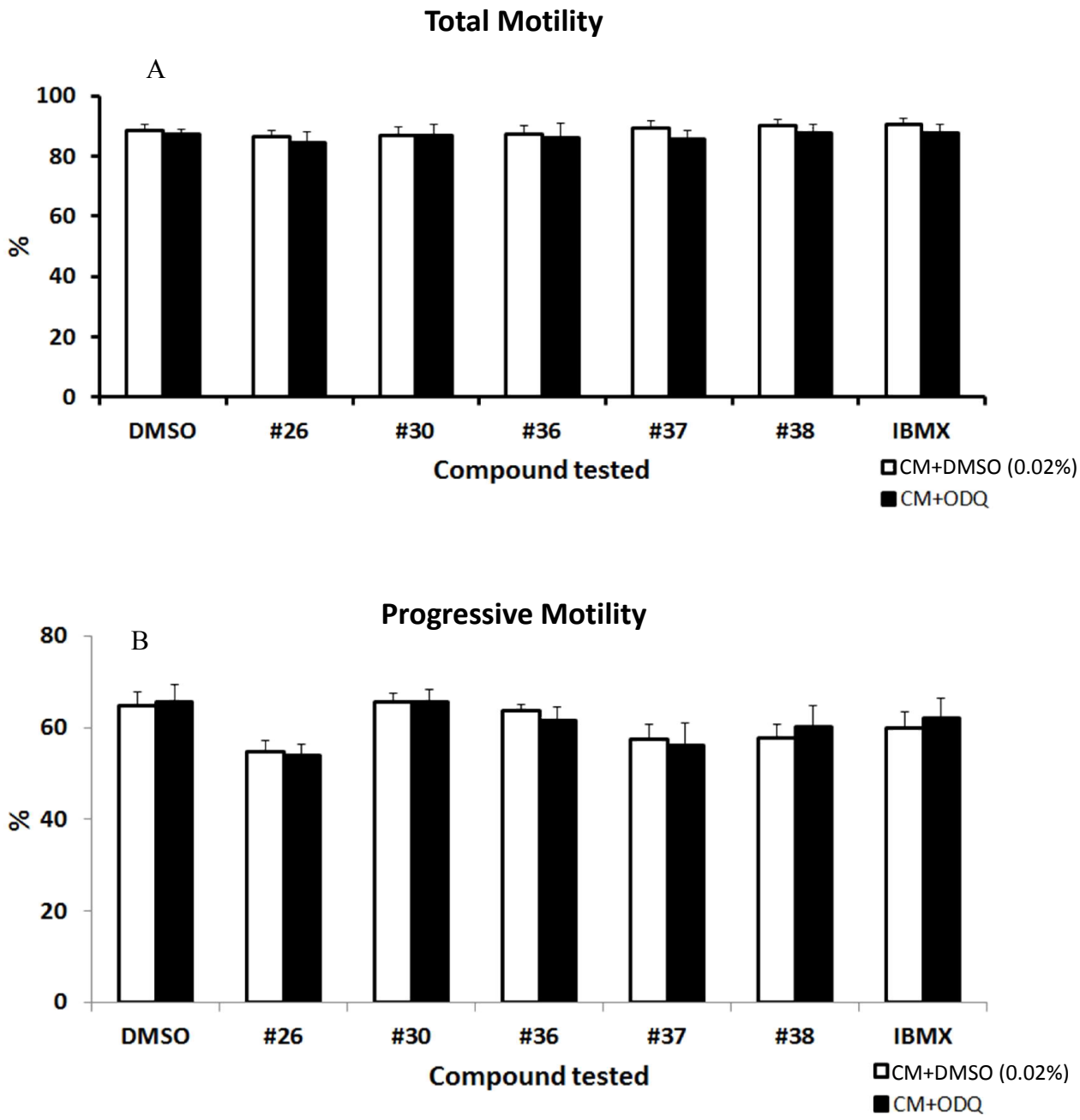


Figure 4-11: Inhibition of sGC with ODQ (under capacitating condition) does not affect total or progressive motility. (A) Total motility and (B) Progressive motility are not significantly different between ODQ (10μM) pre-treated cells and those in CM+DMSO. Values shown were measured 20min after addition of compounds to cell in CM+ODQ and CM+DMSO respectively. ±SEM n=4 (independent experiments): Two Way ANOVA.

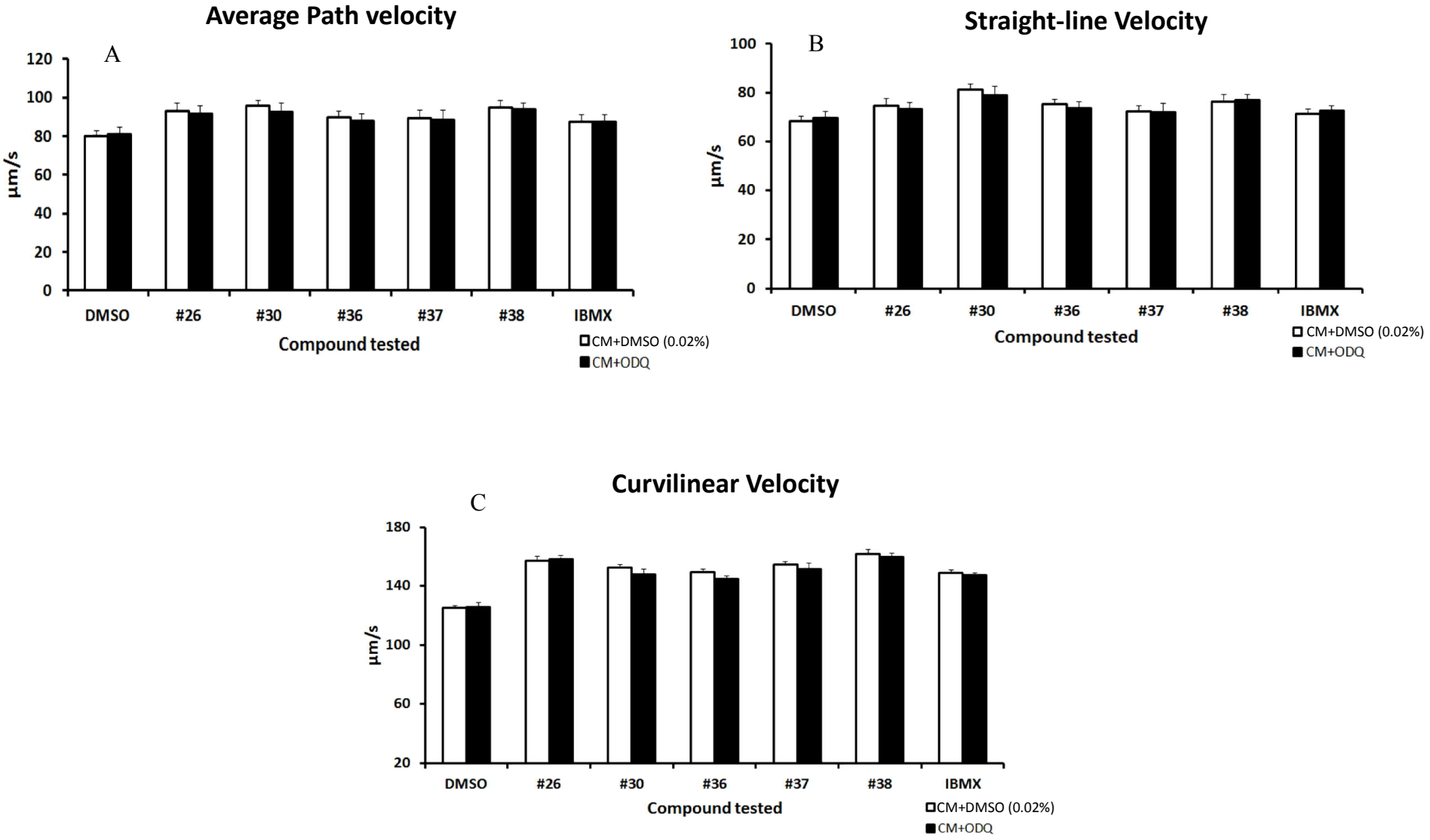


Figure 4-12: Inhibition of sGC with ODQ (under capacitating condition). (A) Average path velocity (B) Straight-line velocity and (C) Curvilinear velocity are not significantly different ($p > 0.05$) in ODQ (10µM) pre-treated cells in comparison to cells in CM+DMSO. Values shown were measured 20min after addition of compounds to cell in CM+ODQ and CM+DMSO respectively. ±SEM n=4 (independent experiments): Two Way ANOVA.

4.4.9 Inhibition of sGC Activity by ODQ does not Affect Velocities of Sperm Cells Incubated under Capacitating Conditions.

Sperm velocities showed a general trend: sperm cells incubated in CM+DMSO are not significantly different (p >0.05) to those incubated in CM+ODQ for VAP, VSL and VCL respectively. Specifically for VAP, when comparing CM+DMSO vs CM+ODQ, there is no statistically significant difference in average VAP (compare white vs black bar chart: Figure 4-12A). Multiple comparison tests also revealed that cells incubated in CM+DMSO or in CM+ODQ are not significantly (p >0.05) different from their respective controls (compare compounds vs DMSO).

There is no significant difference in VSL for each treatment conditions when comparing CM+DMSO vs CM+ODQ (compare white vs black bar chart: Figure 4-12B). The effects of PDEi on the basis of incubation conditions indicated that, for cells in CM+DMSO, only cells treated with compound #30 are significantly (p <0.05) different in VSL when compared with control, other PDEi treated cells are not statistically different to control (compare white bar chart: Figure 4-12B). For cells incubated in CM+ODQ, there is no significant difference between control and PDEi treated samples: p =0.3078 (compare black bar chart: Figure 4-12B).

For curvilinear velocity, sperm cells incubated in CM+DMSO are not significantly different to those in CM+ODQ (p > 0.05) respectively (compare white vs black bar chart: Figure 4-12C). However, when effect of PDEi on cells incubated in CM+DMSO was compared with control samples and each other, there is no significant (p=0.0787) increase in mean VCL was found (compare white bar chart: Figure 4-12C). The mean VCL of sperm cells incubated in CM+ODQ, are not significantly different (p=0.8852) to control (compare black bar chart: Figure 4-12C).

4.4.10 Inhibition of sGC Activity by ODQ does not Affect Hyperactivated Motility of Sperm Cells Incubated under Capacitating Conditions.

The effect of PDEi on hyperactivated motility of cells pre-incubated with or without ODQ was examined under capacitating conditions. Figure 4-13 showed that there is no significant difference in percentage of hyperactivated cells between cells incubation in CM+DMSO or CM+ODQ ($p \geq 0.05$). Multiple comparison tests of cells incubated in CM+DMSO or CM+ODQ also indicates that there is no significant ($p \geq 0.05$) difference in percentage of hyperactivated cells when control and compound treated samples are compared respectively (compare white or black bar chart: Figure 4-13).

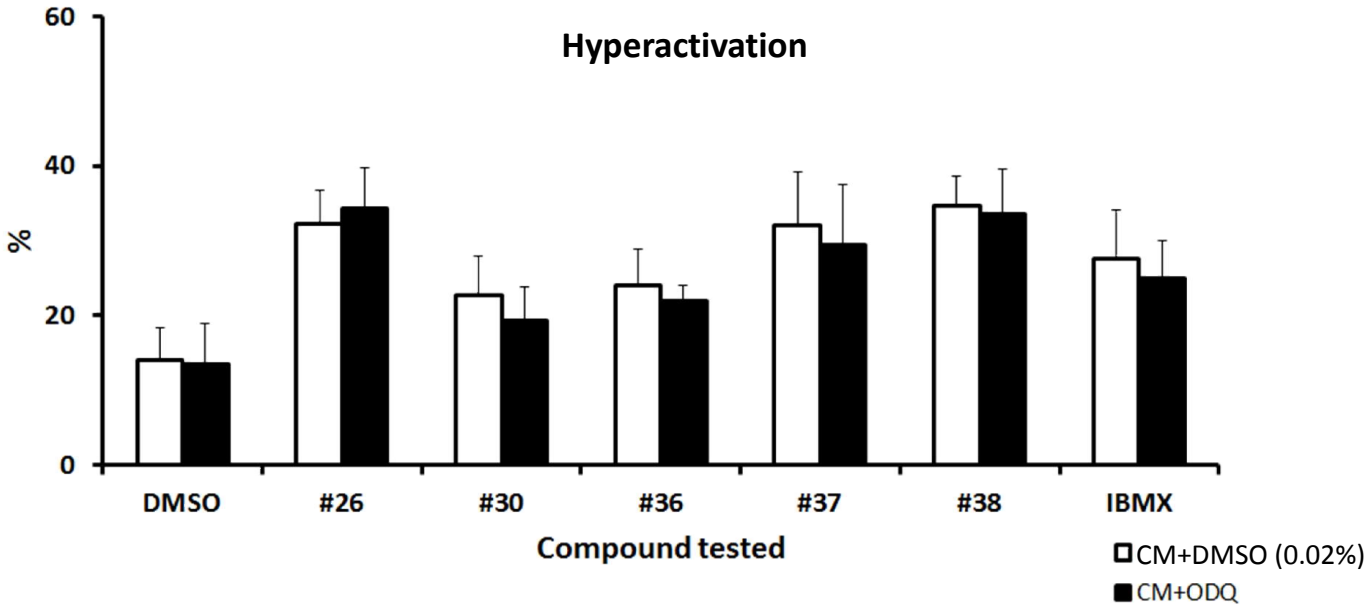


Figure 4-13: Inhibition of sGC with ODQ (under capacitating condition) does not affect percentage of hyperactivated cells. Values shown were measured 20min after addition of compounds to cells in CM+ODQ and CM+DMSO respectively. ±SEM n=4 (independent experiments): Two Way ANOVA

4.4.11 Inhibition of PKG Activity by KT5823 does not Affect Total and Progressive Sperm Motility under Non-capacitating Conditions

In order to characterise the involvement of PKG pathway in effecting the motility enhancing effects of compound #26, #30, #36, #37 and #38, KT5823 (10µM) was used to modulate PKG activity (Figure 4-14).

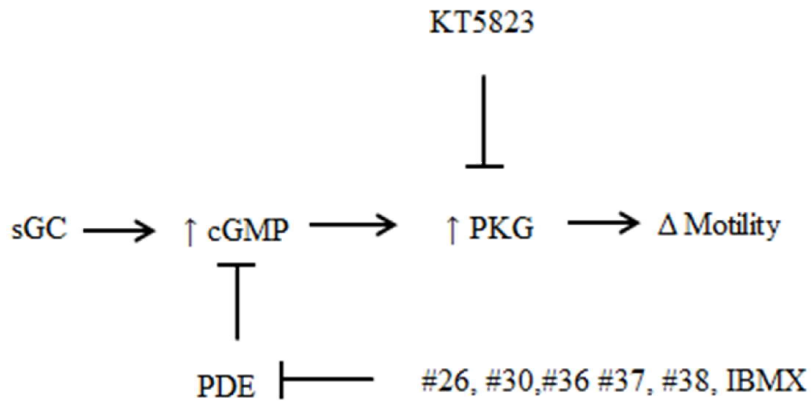


Figure 4-14: Modulation of PKG activity by KT5823 (9*S*,10*R*,12*R*)-2,3,9,10,11,12-Hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3',2',1'-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid, methyl ester (10µM).

Similar experimental conditions to cells treated with ODQ was also used, that is, sperm cells were pre-incubated for 15 min, under non-capacitating conditions and with or without 10µM final concentration of KT5823, before exposure (20 min) to 100µM final concentration of compound(s) (1% DMSO: Vehicle Control and 500µM IBMX: Positive Control) (see Figure 4-15). A two-way factorial ANOVA was conducted on percentage total and progressive

motility, with incubation conditions (with or without KT5823) and treatment conditions (compound treatment) as factors. Figure 4-15A and B showed that there is no significant difference between total and progressive sperm motility when cells incubated in NCM+DMSO and NCM+KT5823 were compared (compare white vs black bar chart: Figure 4-15A and B). This result indicates that pre-incubation of cells in KT5823 (10μM), before exposure to compounds, does not affect total and progressive motility of sperm cells. However, when incubation conditions (that is, effect of compound on sperm cells in NCM+DMSO or in NCM+KT5823) was considered, there is a significant increase in percentage of cells with progressive motility in comparison with respective controls. For cells in NCM+DMSO, post hoc analysis using Tukey's procedure (α level = 0.05), revealed a significant increase in percentage of progressively motile cells in comparison to control (1% DMSO); (as indicated by letter b: #26, #30, #36, #37, #38 and IBMX: $p \leq 0.0001$) (compare white bar chart: Figure 4-15B). Similar pattern of significant increase in percentage of progressively motile cell over control was observed for cells in NCM+KT5823, (as indicated by double hashtag (##): #26, #30, #36, #37, #38 and IBMX: $p \leq 0.0001$) (compare black bar chart: Figure 4-15B). With these results, it is clear that pre-incubation of sperm cells in KT5823 (10μM) does not affect the motility enhancing effects of the compound(s).

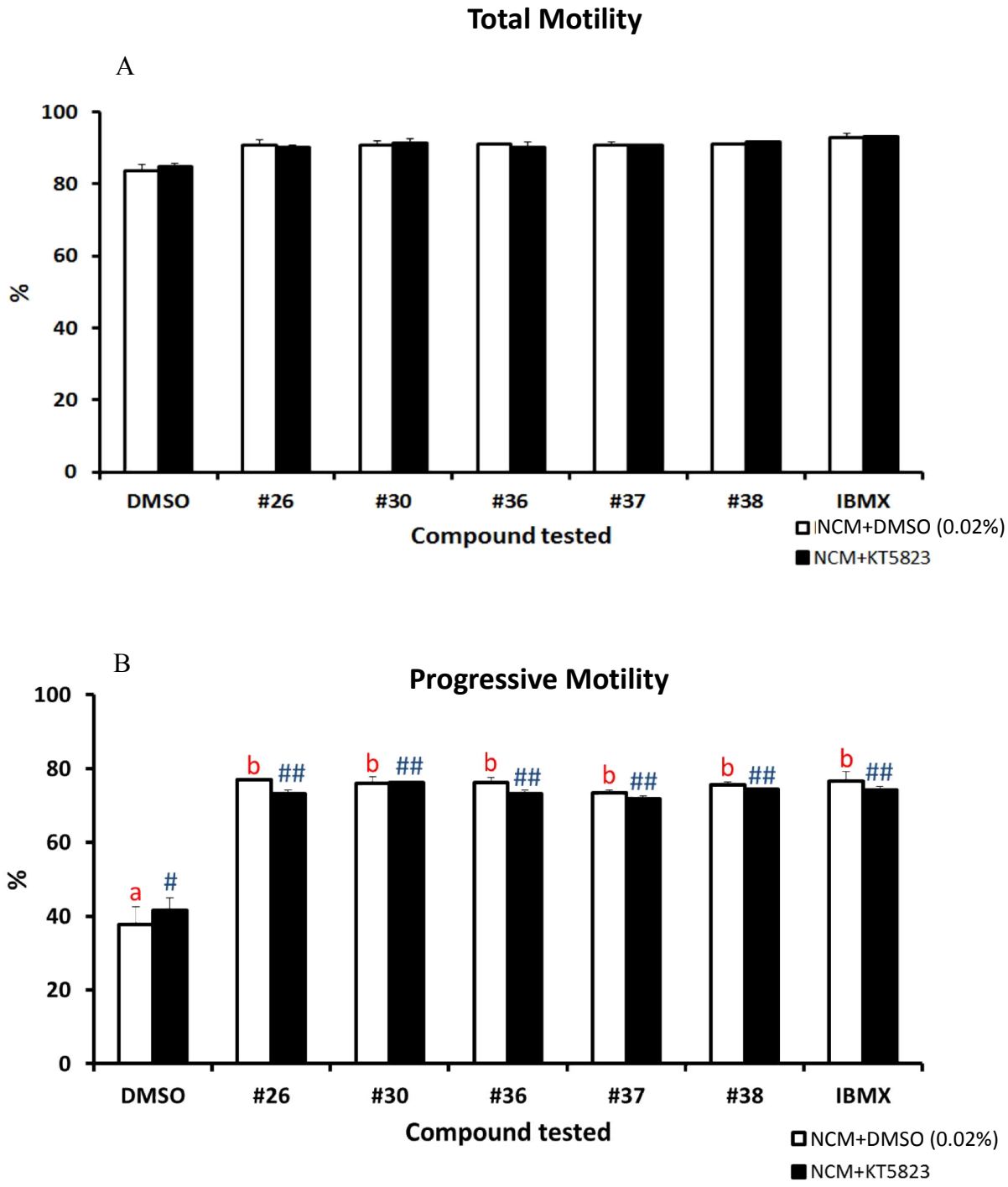


Figure 4-15: Inhibition of PKG with KT5823 (under non capacitating condition) does not affect total or progressive motility. (A) Total motility and (B) Progressive motility are not significantly different between KT5823 (10µM) pre-treated cells and those in NCM+DMSO. Values shown were measured 20min after addition of compounds to cell pre-treated (15min) in NCM+KT5823 and NCM+DMSO respectively. ±SEM n=3 (independent experiments): Two Way ANOVA. There is no significant difference between cells pre-treated with or without KT5823). Difference in a treatment group are represented by different letters (a-b for those in NCM+DMSO: those with different letters differed significantly) and hashtags (# for those in NCM+KT5823: those with double hashtag differed significantly from those with single hashtag.) respectively.

4.4.12 Inhibition of PKG Activity by KT5823 does not Affect Velocities of Sperm Cells Incubated under Non-capacitating Conditions.

When velocities of cells incubated in NCM+DMSO were compared with those in NCM+KT5823 for each treatment conditions, there was no significant difference in average velocities for all the velocities analysed: VAP VSL and VCL respectively (compare white vs black bar chart: Figure 4-16).

Comparison of incubation conditions (that is, effect of compound on sperm cells in NCM+DMSO or in NCM+KT5823) showed that there was a significant increase in average VAP, VSL and VCL in comparison with respective controls. For cells incubated in NCM+DMSO, multiple comparison tests indicates that there is significant (p <0.05) difference in average velocities: VAP - (as indicated by letter b) (#26, #36, #37, #38 and IBMX : $p \leq 0.01$), (#30: $p \leq 0.05$) (compare white bar chart: Figure 4-16A); VSL - (as indicated by letter b) (#26 and #38: $p \leq 0.001$), (#30, #36, #37 and IBMX: $p \leq 0.01$) (compare white bar chart: Figure 4-16B), VCL - (as indicated by letter b) (#26 and #38: $p \leq 0.05$) (compare white bar chart: Figure 4-16C) when control (DMSO) and PDEi treated samples are compared.

For cells incubated in NCM+KT5823, similar pattern of significant increase in average velocities: VAP - (as indicated by double hashtag ##) (#26, #30 and #36: $p \leq 0.01$), (#30, #37, #38 and IBMX : $p \leq 0.05$) (compare black bar chart: Figure 4-16A), VSL - (as indicated by double hashtag ##) (#26, #30 and #36: $p \leq 0.001$), (#38 and IBMX: $p \leq 0.01$) (#37: $p \leq 0.05$) (compare black bar chart: Figure 4-16B), VCL -(as indicated by double hashtag ##) (#26 and #36: $p \leq 0.05$) (compare white bar chart: Figure 4-16C), over control sample were observed for cells incubated in NCM+KT5823.

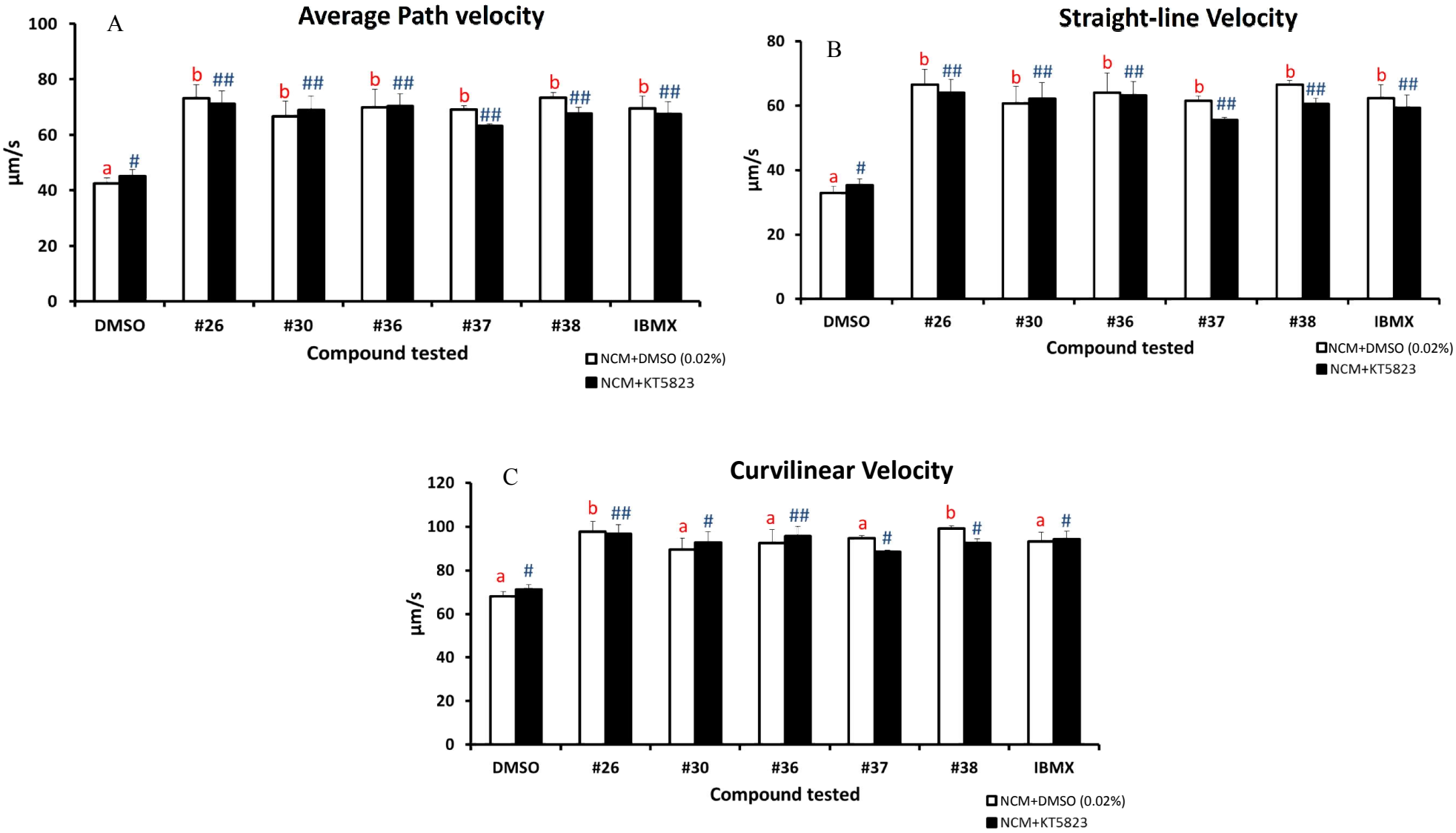


Figure 4-16: Inhibition of PKG with KT5823 (under non capacitating condition). (A) Average path velocity (B) Straight-line velocity and (C) Curvilinear velocity are not significantly different ($p > 0.05$) in KT5823 (10µM) pre-treated cells in comparison to cells in NCM+DMSO. Values shown were measured 20min after addition of compounds to cell pre-treated (15min) in NCM+KT5823 and NCM+DMSO respectively. ±SEM n=4 (independent experiments): Two Way ANOVA. Difference in a treatment group are represented by different letters (a-b for those in NCM+DMSO: those with different letters differed significantly) and hashtags (# for those in NCM+KT5823: those with double hashtag differed significantly from those with single hashtag.) respectively.

4.4.13 Inhibition of PKG Activity by KT5823 does not Affect Total and Progressive Motility of Sperm Cells Incubated under Capacitating Conditions

In this section, the effects of KT5823 on sperm incubated under capacitating conditions were assessed. Sperm cells were pre-incubated with inhibitor (KT5823) in non-capacitating media for 15 min prior to resuspending the cells in capacitating media that contains KT5823 (10μM) (CM+KT5823) and incubation for 3hrs. Alternatively, sperm cells were resuspended in capacitating media (CM+DMSO) and incubated for 3hrs. After 3hrs incubation, cells from each incubation conditions were exposed to compounds for 20 min.

Figure 4-17A and B shows that exposure of sperm cells to KT5823 under capacitating condition has no significant (p >0 .05) effect on total and progressive sperm motility (compare white vs black bar chart: Figure 4-17A and B). Additionally, comparison of incubation conditions (that is, sperm cells in CM+DMSO or CM+KT5823), also indicates that there is no significant difference between control and compound treated samples- total motility CM+DMSO (p = 0.6633 compare white bar chart: Figure 4-17A), CM+KT5823 (p =0.9714 compare black bar chart: Figure 4-17A); progressive motility CM+DMSO (p = 0.6356 compare white bar chart: Figure 4-17B), CM+KT5823 (p =0.8197 compare black bar chart: Figure 4-17B) respectively.

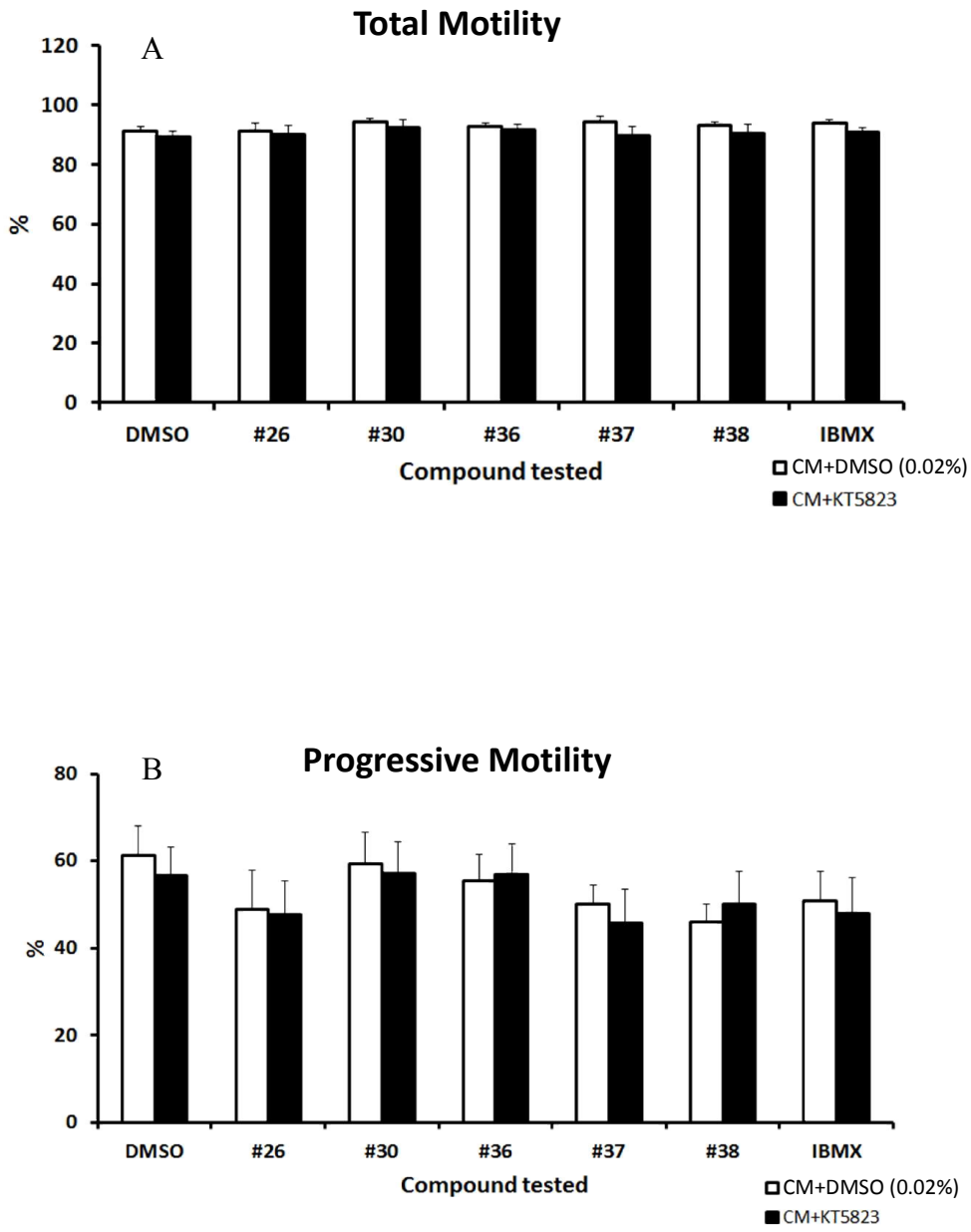


Figure 4-17: Inhibition of PKG with KT5823 (under capacitating condition) does not affect total or progressive motility. (A) Total motility and (B) Progressive motility are not significantly different between KT5823 (10μM) pre-treated cells and those in CM+DMSO. Values shown were measured 20min after addition of compounds to cell in CM+KT5823 and CM+DMSO respectively. ±SEM n=3 (independent experiments): Two Way ANOVA.

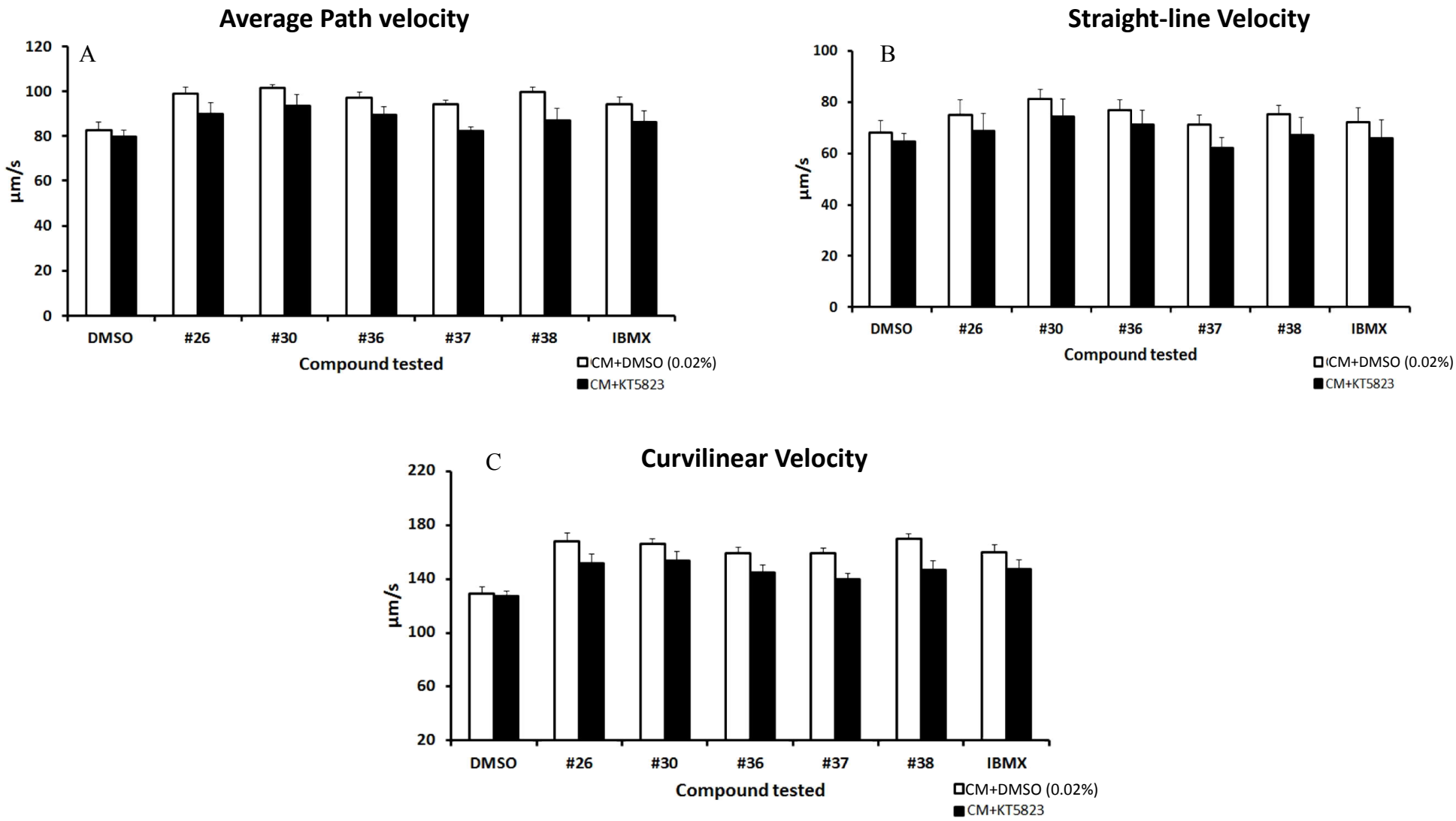


Figure 4-18: Inhibition of PKG with KT5823 (under capacitating condition). (A) Average path velocity (B) Straight-line velocity and (C) Curvilinear velocity are not significantly different ($p > 0.05$) in KT5823 (10µM) pre-treated cells in comparison to cells in CM+DMSO. Values shown were measured 20min after addition of compounds to cell in CM+KT5823 and CM+DMSO respectively. ±SEM n=3 (independent experiments): Two Way ANOVA.

4.4.14 Inhibition of PKG Activity by KT5823 does not Affect Velocities of Sperm Cells Incubated under Capacitating Conditions.

Sperm velocities showed a general trend: sperm cells incubated in CM+DMSO are not significantly different (p >0.05) to those incubated in CM+KT5823 for VAP, VSL and VCL respectively. Specifically for VAP, when comparing CM+DMSO vs CM+KT5823, there is no statistically significant difference in average VAP for each treatment conditions (compare white vs black bar chart: Figure 4-18A). Multiple comparison tests also revealed that cells incubated in CM+DMSO or in CM+KT5823 are not significantly (*p >0.05) different from their respective controls (compare compounds vs DMSO).

There is no significant difference in VSL for each treatment conditions when comparing CM+DMSO vs CM+KT5823 (compare white vs black bar chart: Figure 4-18B). The effects of compounds on the basis of incubation conditions indicated that, for cells in CM+DMSO, cells treated with compound #30 are significantly (*p <0.05) different in VSL when compared with control, other compound treated cells are not statistically different to control (compare white bar chart: Figure 4-18B). For cells incubated in CM+KT5823, there is no significant difference between control and compound treated samples: p =0.3078 (compare black bar chart: Figure 4-18B).

For curvilinear velocity, sperm cells incubated in CM+DMSO are not significantly different to those in CM+KT5823 (p > 0.05) respectively (compare white vs black bar chart: Figure 4-18C). However, when effect of compound(s) on cells incubated in CM+DMSO was compared with control samples and each other, no significant (p=0.0787) increase in mean

VCL was found (compare white bar chart: Figure 4-18C). The mean VCL of sperm cells incubated in CM+KT5823, are not significantly different (p=0.8852) to control (compare black bar chart: Figure 4-18C).

4.4.15 Inhibition of PKG Activity by KT5823 does not affect Hyperactivated Motility of Sperm Cells Incubated under Capacitating Conditions.

The effect of compound(s) on hyperactivated motility of cells incubated with or without KT5823 was examined under capacitating conditions. Figure 4-19 shows that there is no significant difference in percentage of hyperactivated cells between cells incubation in CM or CM+KT5823 ($p \geq 0.05$). Multiple comparison tests of cells incubated in CM or CM+KT5823 also indicated that there is no significant ($p \geq 0.05$) difference in percentage of hyperactivated cells when control and compound treated samples are compared respectively (compare white or black bar chart: Figure 4-19).

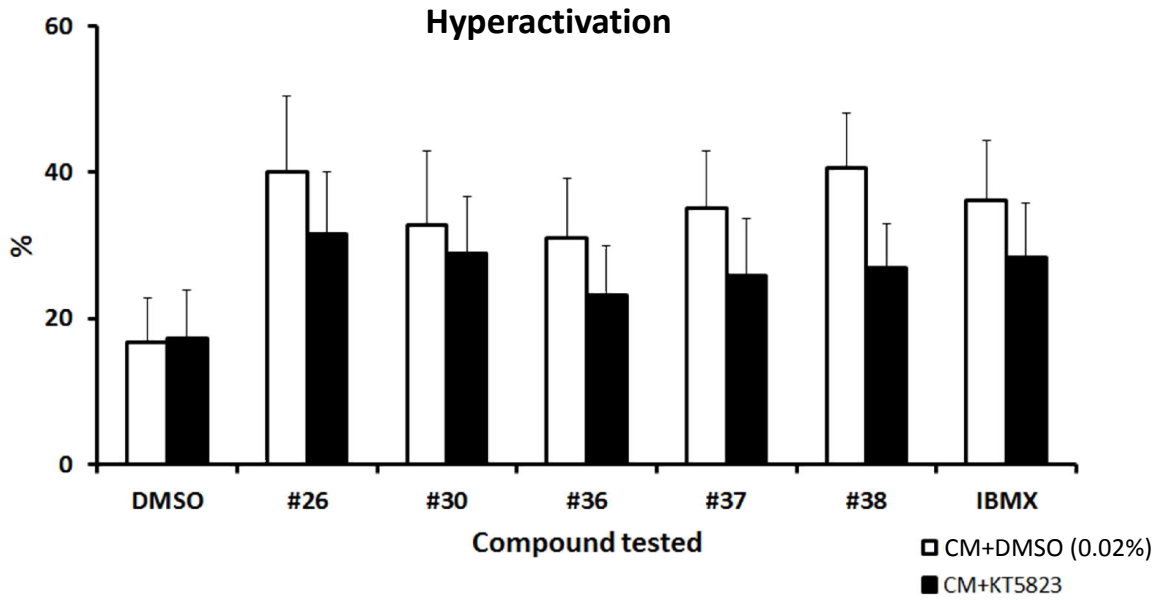


Figure 4-19: Inhibition of PKG with KT5823 (under capacitating condition) does not affect percentage of hyperactivated cells. Values shown were measured 20min after addition of compounds to cells in CM+KT5823 and CM+DMSO respectively. ±SEM n=3 (independent experiments). Two Way ANOVA.

4.5 Discussion

This present study serves to determine the intracellular pathway(s) involved in the motility stimulating effects of the compounds with reported phosphodiesterase inhibitor activity identified in Chapter 3. The effects of PDEi's on sperm motility and velocities were assessed in the presence of chemical compounds capable of inhibiting PKA, sGC and PKG activity respectively. Sperm cells were pre-incubated with or without H89, ODQ and KT5823, under capacitating and non-capacitating conditions respectively, before exposure to PDE inhibitors (1% DMSO: Vehicle Control and 500µM IBMX: Positive Control).

The non-capacitating conditions used in this study support basal but constitutive activation of sAC since media constituent contains Ca^{2+} (1.8mM) and low concentration of Mg^{2+} (0.8mM). Study by Litvin et.al 2003, shows that in the absence of HCO_3^-, activity of recombinant human sAC is only inhibited at high ATP-Mg^{2+} (substrate) concentrations (>6mM) and presence of HCO_3^- intensifies the reaction rate of sAC (30-fold stimulation of activity); thereby increasing the rate of converting ATP to cAMP (Litvin et al., 2003). Therefore, the components of the medium in which the sperm are incubated can have different effects on intracellular enzymes, and so affect motility.

Under non-capacitating conditions and in the absence of PKA inhibitor (H89), PDEi's were effective in enhancing progressive motility and all velocity parameters analysed when compared with their relative controls (compare white bar chart: Figure 4-3B and Figure 4-4). Considering the sAC-cAMP/PKA signalling pathway, these results mean that addition of PDEi's prevents the degradation of cAMP produced as a result of basal activity of sAC. In effect, increase in intracellular cAMP concentration leads to enhanced activation of PKA activity and the resultant enhancement in progressive and motility parameters.

However, pre-incubation of spermatozoa with H89 (50 μM) results in a significant decrease in progressive motility and velocities and addition of PDEi's could not rescue the inhibitory effects of H89 under this condition (Figure 4-3B and Figure 4-4). The inhibitory effects of H89 were not due to non-specific effects on the viability of the spermatozoa, because this reagent had no significant effect on total motility (Figure 4-3A). Additionally, concentrations of H89 ranging from 10 to 100 μM have been used by other studies to assess the role of PKA in sperm motility and capacitation and these studies have shown that H89 is not toxic to human sperm (Aitken et al., 1998a, Bajpai and Doncel, 2003, Battistone et al., 2013). Thus, the decrease in motility is most likely due to an antagonistic effect on PKA; and since addition of PDEi did not rescue decrease in motility and velocities, it indicates that PKA is working downstream of PDEi activity. Additionally the results presented here showed that overall; cAMP/PKA pathway is crucial for basal motility of human sperm.

Interestingly, however, when sperm were resuspended in capacitating media and incubated in the presence of H89 (CM+H89), progressive motility was restored (Figure 4-5B). This result could possibly suggest that there is an alternative pathway in spermatozoa, independent of PKA, which supports progressive motility under capacitating conditions even if cells were pre-treated / exposed to PKA inhibitor: H89. In many cellular systems, there are often complex signal transduction pathways: involving cross-talk between effector feedback inhibition mechanisms, and compensatory mechanisms that are vital for the dynamic and adaptive nature of cellular responses (Bajpai and Doncel, 2003, Logue and Morrison, 2012). Indeed, regulation of mammalian sperm motility is a complex process involving activation and/or down regulation of different signal transduction pathways (Thundathil et al., 2002, Turner, 2005). In addition to PKA activities, stimulation of sperm motility by Ca^{2+}/CaM/CaM Kinase signalling pathway has been reported by many studies (Lasko et al., 2012, Schlingmann et al., 2007, Ignotz and Suarez, 2005, Marin-Briggiler et al., 2005). In human

and stallion sperm, CaM kinase IV and II has been found to be localised in the principal piece of the flagellum (Marin-Briggiler et al., 2005) and its been suggested to achieve some of the effects of Ca^{2+} on the flagellum; since inhibition of CaM decreases sperm motility (Si and Olds-Clarke, 2000, Ignotz and Suarez, 2005, Lasko et al., 2012). Although not investigated in this study, it is probable that Ca^{2+}/CaM/CaM Kinase signalling pathway might be involved in the restoration of progressive motility of sperm cells incubated in the presence of PKA inhibitor: H89.

An alternative argument could be that, resuspending cells in capacitating media that contains HCO_3^- leads to increased sAC activity (30-fold stimulation of activity) and a massive cAMP increase leads to pronounced activation, by inducing dissociation of the catalytic domain from its regulatory subunits, of PKA activity that overcomes H89 inhibitory effect. Indeed, while several studies have used H89 as potent and specific inhibitor of PKA, H89 is a reversible competitive inhibitor of PKA. H89 works through competitive blockade of ATP binding site on PKA catalytic subunit. Thus, increase in the release of PKA catalytic subunit, as a result of rise in cAMP, means H89 and ATP are competing at higher concentration of PKA catalytic subunit and there are chances that ATP binds to some PKA catalytic subunits and then phosphorylate appropriate serine or threonine residues on target proteins that supports progressive motility under this condition of treatment.

In this study, inhibition of PKA activity by H89 suppresses hyperactivated motility; a movement pattern characterised by asymmetrical, high amplitude beats of the flagellum (Figure 4-7) and addition of PDEi was unable to restore hyperactivated motility. This result is in keeping with the proposed role of PKA in sperm; PKA has been shown to phosphorylate several proteins on Ser and Thr residues, activating, either directly or indirectly, several protein kinases and/or inhibiting protein phosphatases, which will finally produce an increase in the total protein tyrosine phosphorylation (Okamura et al., 1985, Bajpai and Doncel, 2003,

Luconi et al., 2005). Therefore, PKA inhibition by H89 blocks the onset of tyrosine phosphorylation which is important for the development of hyperactivation (Visconti et al., 1995b, Nolan et al., 2004a, Naz and Rajesh, 2004, Leclerc et al., 1996a). Additionally, all velocity parameters analysed were found to be significantly reduced when cells were exposed to H89, under capacitating conditions (Figure 4-6). This could be as a result of decreased tyrosine phosphorylation of flagellar proteins which probably leads to decreased flexibility of the fibrous sheath of the sperm tail (Si and Okuno, 1999). Consequently, this result highlights the participation of PKA pathway in enhancing sperm velocity and modulation of hyperactivated motility in human sperm. Additionally, since treatment with PDEi cannot rescue the inhibitory effects of H89, it can be concluded that PKA activity is acting downstream of PDEi's in the signalling pathway.

To establish a role for sCG-cGMP/PKG signalling pathways in human sperm motility and to determine if this pathway is involved in the motility enhancing effects of the PDEi's, activity of sGC and PKG were modulated by ODQ and KT5823 respectively. In the literature, there are still controversies regarding the role of sGC in relation to human sperm motility. Although immunoblotting confirms a low but functional presence of sGC in human sperm, presence of PKG has not been detected in human sperm (Willipinski-Stapelfeldt et al., 2004). Assessment of functional activity of sGC in intact and membrane fraction of human sperm cells, showed that sGC has a relatively low activity in comparison to sAC and atrial natriuretic peptide and related peptides: brain natriuretic peptide and C-type natriuretic peptide are ineffective in elevating sperm cGMP; only sodium nitroprusside (SNP), a nitric oxide (NO) donor, induces a 2.8-fold increase in the relatively low cGMP concentration in human sperm (Revelli et al., 2001, Wang et al., 2014). As a free radical, many investigators support the notion that physiological concentration of NO (<1μM) is beneficial to some sperm functions and increased level of NO is detrimental to sperm cells (Salvolini et al., 2012, de Lamirande et al.,

1997a, Balercia et al., 2004, Lewis et al., 1996, Zini et al., 1995). In a recent study by Miraglia et. al, it was suggested that NO stimulates human sperm through activation of PKG pathway (Miraglia et al., 2011). This study is in contrast to study that suggested that there is no PKG in human sperm (Willipinski-Stapelfeldt et al., 2004). A careful consideration of the incubation conditions used in this study shows that sperm cells were isolated through swim-up technique (for 2hr) in a sperm wash media that contains HCO_3^- (Miraglia et al., 2010). Thus, for this study, the activity of sAC-cAMP/PKA driving the observed effect cannot be ruled out since incubation of cells for 2hrs in media that contains HCO_3^- will lead to activation of this pathway.

In other studies, Sildenafil a type-specific inhibitor of PDE5 that blocks degradation of cGMP has been used to stimulate sperm motility. Both Lefièvre et. al and Cuadra et. al suggested that addition of Sildenafil enhances sperm motility in vitro (Lefièvre et al., 2000, Cuadra et al., 2000). However, sperm preparation methods used by both studies also involves exposure of sperm cells to conditions that could stimulates sAC-cAMP/PKA pathways (Lefièvre et al., 2000). In the study by Lefièvre et. al, a concomitant increase in cAMP level was also reported when Sildenafil (at 100-200μM) is added to sperm. The concomitant increase in cAMP and cGMP highlights a possible cross-talk with cAMP and cGMP signalling pathways. Alternatively, this could suggest that Sildenafil might be non-specific in its action at high concentration used in this study.

Data from this study shows that inhibiting the activity of sGC (ODQ 10μM) and PKG (KT5823 10μM) has no effect on sperm motility. Under non-capacitating conditions (in the absence of HCO_3^-) and in the presence of chemical inhibitor of sGC and PKG, PDEi's were effective in enhancing progressive motility (Figure 4-9B and Figure 4-15B) and there is no difference between cells pre-incubated with or without chemical inhibitor of sGC and PKG respectively. In the same way, PDEi's strongly increase the individual parameters of sperm

velocities (Figure 4-10 and Figure 4-16). This results are totally in contrast to what was observed when cells were pre-incubated with PKA inhibitor (H89), were addition of H89 significantly reduces progressive motility and sperm velocities; and addition of PDEi's could not rescue the inhibitory effect of H89 (see above).

Also, under capacitating conditions (in the presence of HCO_3^-), there was no difference between ODQ and KT5823 treated and untreated sperm cells; addition of compounds resulted in similar motility and velocity pattern for both treated (ODQ and KT5823) and untreated cells: progressive motility (compare white vs black bar chart: Figure 4-11and Figure 4-17), sperm velocities (compare white vs black bar chart: Figure 4-12 and Figure 4-18) and hyperactivated motility (compare white vs black bar chart: Figure 4-13 and Figure 4-19). Since inhibition of sGC and PKG activity did not blunt or abolish the effects of PDEi on sperm motility, it can be concluded that sGC and PKG do not participate in the overall signalling pathway involved in the motility enhancing effects of PDEi identified.

In conclusion, this experimental strategy indicated that PKA signalling pathway is mediating the intracellular pathway(s) involved in the motility stimulating effects of PDEi identified Chapter 3. Furthermore, test of sGC and PKG inhibitors showed that motility enhancement by PDEi was independent of sGC and PKG activity. Also, for the first time, data from this study shows that PKA activity is essential for the maintenance of basal progressive motility of human sperm under non-capacitating conditions. In addition, this study also suggested that there might be an alternative pathway independent of PKA that supports progressive motility under capacitating conditions. Finally, data presented here also showed that continuous activation of PKA is necessary for hyperactivated motility.

Chapter 5.

Ibudilast enhances in vitro fertilization rate of in vitro mature porcine oocytes

5.1 Introduction

The overall goal of screening for new compounds in this study is to identify compounds that could be used clinically: as a result of enhancement in sperm motility and functionality in relation to in vitro fertilization. Thus, to build on chapters 3 and 4, animal IVF system was developed in this study as a proof of concept model that demonstrates that addition of PDEi's could enhance IVF. Current ethical approval preclude the use of human oocyte for research, thus, porcine IVF system was used.

Advances in in vitro maturation (IVM) and in vitro fertilization (IVF) of porcine oocyte means use of this model for basic biomedical research purposes is gaining popularity. It has been demonstrated that cells harvested from unused organs from slaughterhouses, such as oocyte, oviductal epithelial cells, cumulus cells (CCs), and granulosa cells, can be grown in culture conditions (Areekijseree, 2003, Areekijseree and Vejaratpimol, 2006, Areekijseree and Veerapraditsin, 2008). These cells have also been shown to be an excellent model for the biological toxicity study of mammalian reproductive system (Sanmanee and Areekijseree, 2009, Sanmanee and Areekijseree, 2010).

Historically, addition of phosphodiesterase inhibitor (such as caffeine) is known to increase the ability of spermatozoa to fertilize porcine oocytes (Niwa, 1993). Previous research has shown that addition of caffeine (concentration ranging from 1- 5mM) to fresh or frozen-thawed ejaculated boar spermatozoa significantly influence oocytes penetration in vitro (Abeydeera and Day, 1997b, Funahashi et al., 2000, Wang et al., 1991, Funahashi and Romar, 2004). By stimulating capacitation and/or acrosome reaction related events during in vitro co-culture of sperm-oocytes, caffeine is thought to increase the chance of more spermatozoa binding to the zona pellucida, thus more sperm cells achieve penetration within a narrow window (Funahashi et al., 2000). However, a high incidence of polyspermic

penetration (the penetration of an oocyte by more than one spermatozoon) remains a persistence problem to porcine IVF systems when caffeine is added to culture media (Machatkova et al., 2007, Niwa, 1993, Funahashi and Day, 1997, Day, 2000).

Examining the effect of caffeine (1mM), fertilization-promoting peptide (FPP) (100nM), and adenosine (10μM) on fertilization parameters of in vitro matured oocytes, Funahashi and colleagues report that a significantly higher proportion of oocytes were penetrated when IVF medium contained caffeine (98%) in comparison with FPP (75%) and adenosine (71%) respectively (Funahashi et al., 2000). Nevertheless most of the oocytes were polyspermic in the presence of caffeine (87%) when compared with FPP (25%) or adenosine (21%) (Funahashi et al., 2000). In another study comparing in vitro fertility of boars with high field fertility (Machatkova et al., 2007), it was reported that treatment of spermatozoa with caffeine leads to significant polyspermic rate (range: 50-92%) in comparison to untreated samples (range: 61-75%). This high incident of polyspermic penetration has been attributed to slow zona reaction as well as simultaneous penetration by a number of spermatozoa with induced acrosome reaction as a result of caffeine in fertilization medium (Wang et al., 1998, Han et al., 1999, Funahashi and Nagai, 2001). Thus, an ideal compound that will support/enhance in vitro porcine IVF system would: (1) enhance capacitation related events without significant induction of acrosome reaction, (2) not increase polyspermy rate or affect subsequent development and (3) be non-toxic to both sperm and oocyte respectively.

5.2 Aims and Experimental Design

The aim of this chapter is to determine if addition of compound #26 (Ibudilast) during in vitro fertilization will promote penetration of ejaculated spermatozoa into oocytes. To achieve

this, in vitro matured porcine oocytes were fertilized in vitro and rate of fertilization determined. Additionally, the effect(s) of this compound on boar sperm motility and velocity will be investigated using CASA, to determine if the compound produced the same effect as shown in human in Chapter 3 and Chapter 4.

5.3 Materials and Methods

5.3.1 Chemicals and Media

The oocyte maturation media (OMM) was a TCM 199- based medium consisting of TCM 199 salts (TCM-199 with Earle′s salts and L-glutamine, without sodium bicarbonate, Sigma-Aldrich, UK) supplemented with 26.2mM $NaHCO_3$, 0.9mM Sodium pyruvate, 1% BSA (heat inactivated), 0.1μg/ml luteinizing hormone (LH), 0.1μg/ml Follicle stimulating hormone (FSH), 4ng/ml Epidermal growth factor (EGF), 10% (v/v) porcine follicular fluid (pFF), 50μg/mL streptomycin sulphate, and 50 IU/mL penicillin. The follicular fluid was the pooled aspirate of healthy follicles, 6–8 mm diameter; the pooled fluid was centrifuged at 2500 g for 20 min to remove cell debris, filtered through 0.22 mm membrane filters and stored at -20 degrees Celsius until needed. The oocyte collection media (OCM) was a TCM 199 medium (TCM 199- With Hanks′ salts and L-glutamine, without sodium bicarbonate, Sigma-Aldrich, UK), supplemented with 4.2 mM $NaHCO_3$, 10 mM HEPES and 1 mg/mL polyvinyl alcohol (PVA) and it was used for washing the oocytes. Media's pH was adjusted to 7.4 with 1M NaOH and was filtered through 0.22 mm disposable filtration unit under sterile conditions.

The in vitro fertilization media (IVF-m) was based on a modified Tris-buffered media described by (Ye et al., 2007) with slight modifications. IVF-m consist of 113 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2.2H_2O$, 20 mM Tris-base, 11 mM glucose, 5 mM sodium pyruvate,

0.3% (w/v) BSA, 0.2 mM freshly prepared reduced glutathione (antioxidant), but free of antibiotics (pH 9.9 at 4^{o}C, balanced to pH 7.4 at 39^{o}C in 5% CO_2 for 12–24 h). All media used in this study were equilibrated beforehand to the gas phase and temperature at which they were to be used.

5.3.2 Ovary Collection and In Vitro Maturation of Oocytes

Pre-pubertal pig ovaries were collected from a local commercial abattoir. They were removed within 30 min of slaughter and transported to the laboratory within 1 hour. Selected ovaries were washed thrice in sterile phosphate buffered saline (PBS). Ovaries were then placed in a sterile Petri dish containing 3ml of OCM. The follicular contents of selected healthy (with a translucent appearance and extensive vascularisation) follicles with diameter of about 3–8 mm were released into the OCM, in the Petri dish, by making an incision (with a scalpel) in the follicles. Cumulus oocyte complexes (COCs) with more than three intact and compact cumulus layers were selected, under a stereomicroscope, for culture after washing in fresh OCM. Then, groups of 25–40 COCs were transferred into OMM (500µl) and incubated under an atmosphere of 5% CO_2 in humidified air at 39 °C for 48h. All culture containing oocytes were covered with a thin layer of pre-equilibrated (5% CO_2 in humidified air at 39 °C) mineral oil (500µl). Incubator temperatures and gas remained constant of their expected values except for short periods when doors were opened to access cells.

5.3.3 Preparation of Spermatozoa and In Vitro Fertilization (IVF)

Fresh extended pig semen (Pig Improvement Company UK Limited) stored up to 2 day at 16-17^{o}C in TRIXCell extender (IMV technologies) was washed twice by centrifugation (5 min, 500 g) in IVF-m. The sperm pellet was resuspended in IVF-m and sperm concentration and motility characteristics determined by CASA. The sperm suspension was pre-incubated for a

short period (10 min) at 39^{o}C before co-incubation with oocytes. At the end of maturation, COCs were denuded of cumulus cells by brief vortexing (1–2 min) in warm OMM (balanced at 39^{o}C in 5% CO_2), washed in the same media and transferred to fresh dish containing 500µl IVF-m which were covered with mineral oil and pre-incubated for 2-3 h. To fertilize the oocytes, 10 µl aliquot of sperm cells (1×10^5 sperm cells/ml) was added to OMM culture media containing in vitro matured oocytes. After addition of DMSO (Vehicle control) and compound #26 (1% v/v and 100µM final concentration respectively), oocyte–sperm co-incubation was carried out for 3 h at 39^{o}C under 5% CO_2 in humidified air. After a brief wash in IVF-m, putative fertilized oocytes were cultured in IVF-m for 18h.

5.3.4 Fixing and Staining of Fertilized Oocytes

To ensure fertilized oocytes can be observed at the pronuclei (2PN) stage, the inseminated oocytes were (after 18h) washed by gentle pipetting in fresh IVF-m to remove loosely attached spermatozoa and cumulus cells. The oocytes were then permeablized in 100% ice-cold methanol solution (-20^{o}C) for 1h. The nucleic acid of permeablized oocytes was then stained with Hoechst stain (Hoechst 33342 Sigma: 3µg/ml) for 30-45min at 4^{o}C. Fertilization was independently assessed and the number of fertilized oocytes (oocytes with 2PN) was scored using a fluorescent microscopy.

5.3.5 Fertilization Assessment

Fertilization stage of oocytes was assessed using a fluorescence microscope. Oocytes were determined to be penetrated when male pronucleus (pronuclei) were found. Oocytes with more than one male pronucleus were assessed as polyspermic. Only oocytes with one male and one female pronucleus were determined as monospermic.

5.3.6 Boar Sperm Treatment and Motility Analysis

The CASA analysis was performed by using a commercial system (SCA® Microptic version 5.1.0.1, S.L., Barcelona, Spain). In this procedure, compound (#26: 100μM) and DMSO (1% v/v) was added to samples that were previously warmed at 37°C for 10 min. 5 μL aliquots of these samples were then placed on a warmed (39°C) microscope and covered with a 25- mm x 25-mm coverslip slides (glass slides and coverslips were coated with 0.4% agarose). Sperm motion analysis was based on the examination of 30 consecutive, digitalized images obtained from a single field using a 10× positive phase contrast objective. Images were taken with a time lapse of 1s - the image capture speed was therefore one every 33 millisecond. Three to four separate fields were taken for each sample. With respect to the setting parameters for the SCA® program, a sperm cell with a VAP < 20 μm/s was considered static or immotile, while objects with a velocity >20 μm/s were considered motile. Sperm cells with >45% STR (Straightness coefficient: (VSL/VAP) x100) and with velocities between 30 and 45 μm/s were considered as sperm with slow progressive motility speed; those with a velocity >45μm/s were considered as sperm with fast progressive motility (Ramio et al., 2008, Broekhuijse et al., 2011).

5.3.7 Statistics

The data were expressed as mean percentages ± SEM of penetrated oocytes from inseminated oocytes, and polyspermic and monospermic oocytes from penetrated oocytes. For motility analysis, all data are presented as means ± SEM. The statistical analysis was carried out using GraphPad Prism 6 (GraphPad Software, California, USA). The data were analysed by One-way ANOVA on paired observations. Differences were considered significant at a $P < 0.05$.

5.4 Results

5.4.1 In Vitro Maturation (IVM) of Porcine Oocyte

The presence of cumulus cells is considered a significant element of the cumulus oocyte complex that supports in vitro nuclear and cytoplasmic maturation necessary for successful male pronuclear (MPN) formation and developmental competence of oocytes. In this study, oocytes selected for in vitro maturation are based on the characteristic of the cumulus cells surrounding the oocytes. Oocytes with no cumulus cells (denuded oocytes) or those with partial cumulus cell layers were not selected for IVM (Figure 5-1A and B). Oocytes with intact-cumulus, with ≥ 5 layers of compact cumulus cells, and/or those with multi-layered cumulus, with 2–3 incomplete layers of CCs, were matured in vitro, in the presence of 10% (v/v) pFF, EGF, FSH and LH, to produce oocytes with expanded cumulus cell layer (Figure 5-1C and D).

5.4.2 Effects of Ibudilast (Compound #26:100μM)) on Fertilization during Co-incubation

Boar sperm and in vitro matured oocytes were co-incubated in IVF-m in the presence or absence of compound #26 (100 μM) for 3hrs and then putative fertilized oocytes were transferred into fresh sperm free IVF-m for 18h. The presence of 100μM compound #26 during the 3hrs co-culture period significantly improved the rates of sperm penetration (39 ±4% vs 8 ±3%; $p<0.005$) (Table VII). Considering the effect of compound on the rate of monospermic and polyspermic penetration, in total penetrated oocytes examined, 94 ±4% of penetrated oocyte showed monospermic penetration, presence of two pronuclei (2PN) (Table VII) (Figure 5-2A), and 6 ±4% polyspermic penetration, presence of three or more pronuclei (Figure 5-2C), respectively. Oocytes with one pronucleus (PN) were considered unfertilized

(Figure 5-2B). For oocytes in control condition (i.e. treated with DMSO), all eight penetrated oocytes showed monospermic penetration (Table VII).

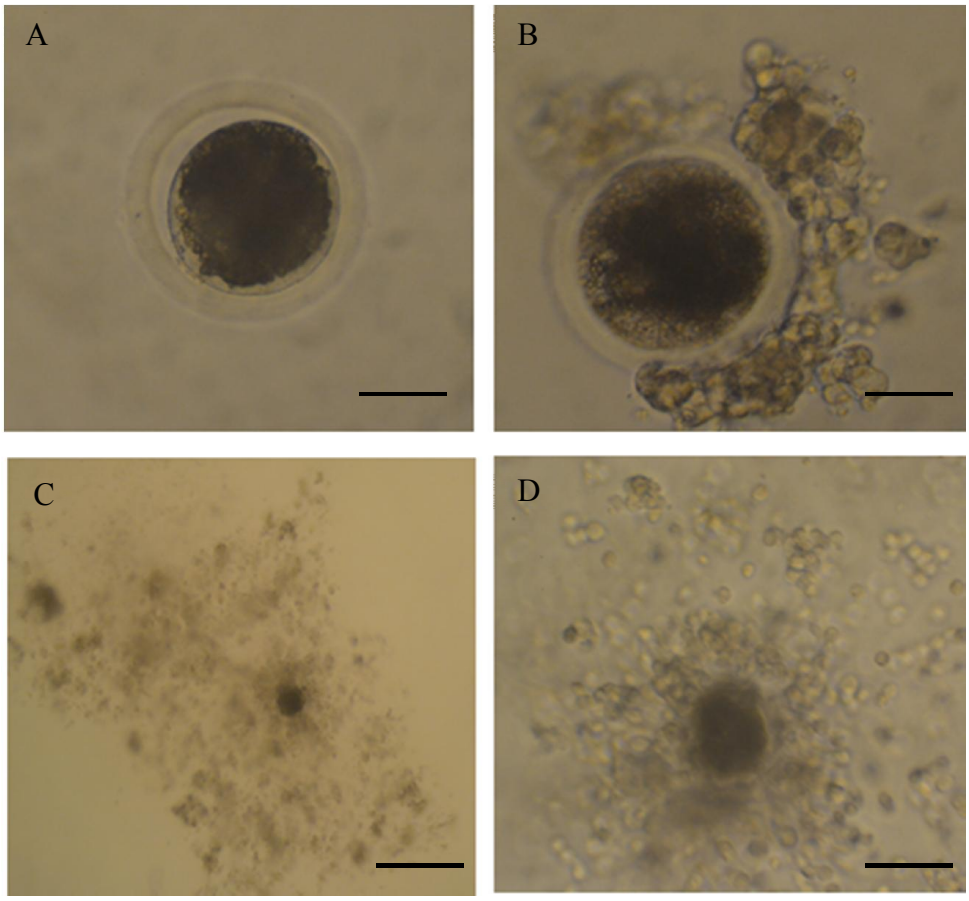


Figure 5-1: Micrographs of an oocyte with a completely denude oocyte (A), an oocyte with partial cumulus cell layers (B), an oocyte with expanded cumulus cell layers (lower magnification) (C), an oocyte with expanded cumulus cell layers (higher magnification) (D). Micrographs are representation of different oocytes assessed using light microscope. The scale bar represents: A and B: 60 µm, C and D 150 and 100 µm respectively.

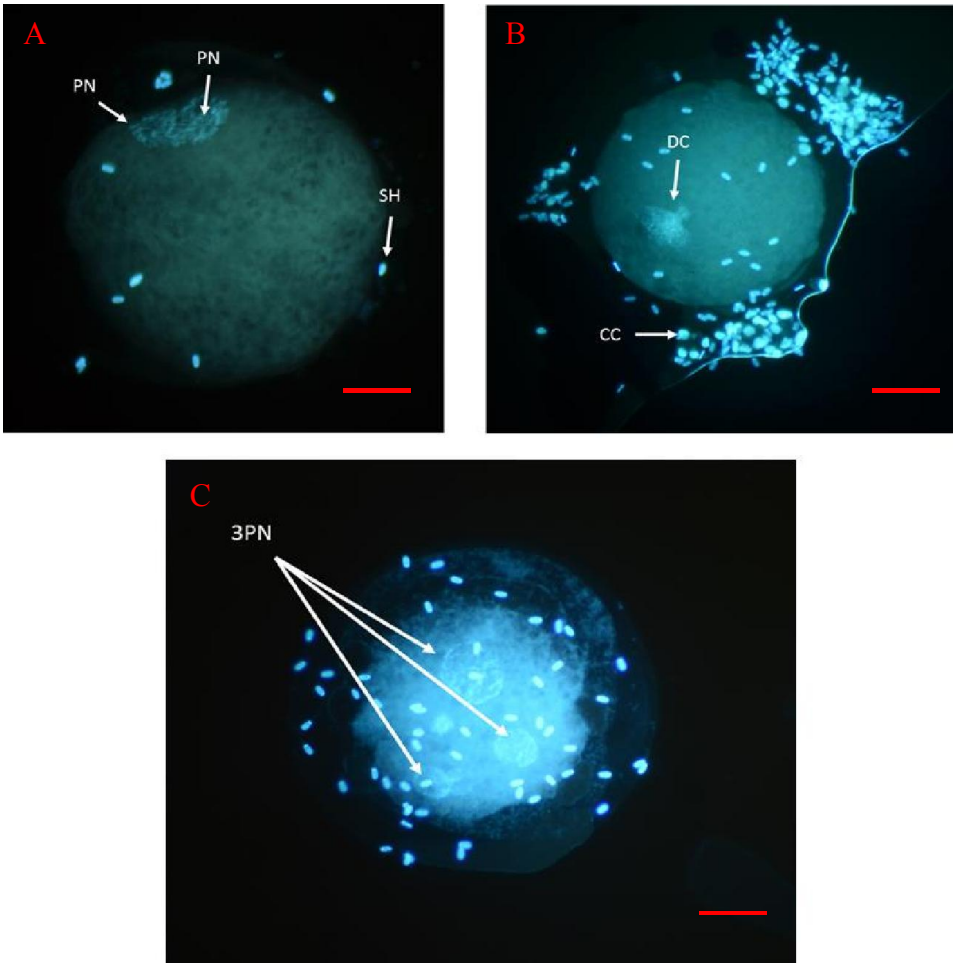


Figure 5-2 Micrograph of hoechst stained porcine oocytes 18 h after the addition of sperm to the oocytes for IVF. In A) a normally fertilized oocyte is seen, with two polar bodies. In B), an oocyte with one pronucleus. In C), polyspermic fertilization has taken place and multiple decondensed sperm head can be seen. PN: Pronuclei. 3PN: 3 Pronuclei. DC:Decondensed Nuclei. CC: Cummulus cell. SH: Sperm Head. Micrographs are representation of different oocytes assessed using fluoresnce microscope. The scale bar represents 40 µm.

Table VII: Effect of Ibudilast (compound #26) on sperm penetration into oocytes in vitro

		% of Oocytes ± SEM		
Treatment	No. of oocytes examined	Penetrated	Monospermy	Polyspermy
DMSO	86	8 ±3[a]	100	-
#26	159	39 ±4[b]	94 ±4	6 ±4

Data are from four replicates.
Percentage of penetrated oocytes is relative to the number of examined oocytes.
Percentage of monospermic oocytes is relative to the number of penetrated oocytes.
[a,b] Values with different superscripts within each column are significantly different (p<0.05).
n=4(independent experiments)

5.4.3 Effect of Ibudilast (Compound #26: 100 µM)) on Boar Sperm Motility

To determine the effects of the compound on boar sperm motility, compound was added to samples that were previously warmed at 37^{o}C for 10 min and motility was assessed at t0, t10 and t20 respectively. As shown in Figure 5-3A, there is no significant difference between total motility, at each time point analysed, when cells incubated in compound #26 (100µM) and DMSO were compared (compare white vs black bar chart: Figure 5-3A). With respect to progressive motility, addition of compound caused a significant increase in the percentage of progressively motile sperm at cells t0 ($p< 0.005$); however, no significant difference was found at other time points (that is t10 and t20) when cells treated with compound #26 and DMSO were compared (compare white vs black bar chart: Figure 5-3B). When velocities of cells treated with compound were compared with those treated with DMSO, there is no significant difference in average velocities for all the velocities analysed and at each time points: VAP: ($p>0.05$) Figure 5-4A; VSL: ($p>0.05$) Figure 5-4B and VCL: ($p>0.05$) Figure 5-4C respectively.

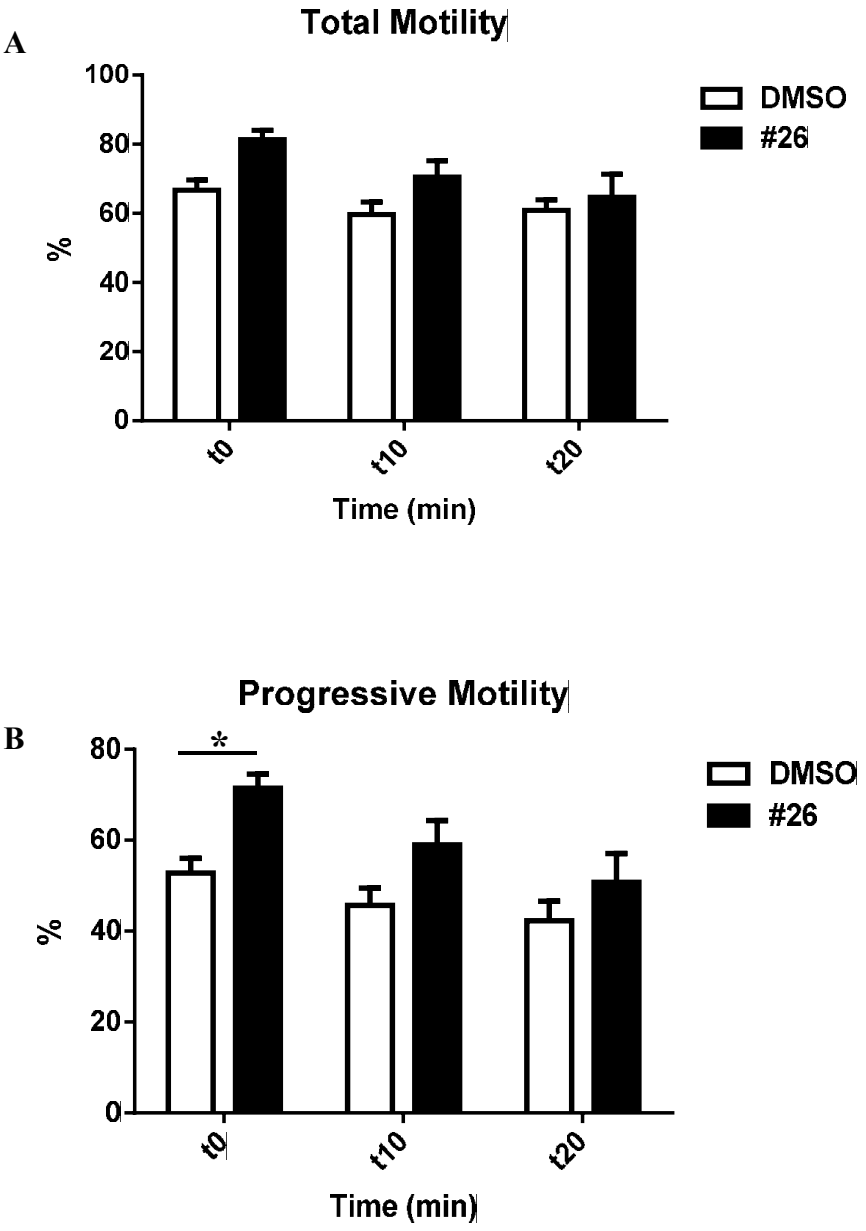


Figure 5-3: Effect(s) of compound (#26) on sperm motility: Boar spermatozoa were treated for 20min at 37^{o}C with 100µM of compound #26 or DMSO (1% v/v). (A) Total motility is not significantly different between DMSO and compound (#26) treated cells at all time-points (t0, t10 and t20 respectively). (B) Progressive motility is significantly increased at t0, however, there is no significant difference between DMSO and compound (#26) treated cells at t10 and t20 respectively. n=7 (7 independent experiments), mean ±SEM. Two Way ANOVA: *$p \leq 0.05$.

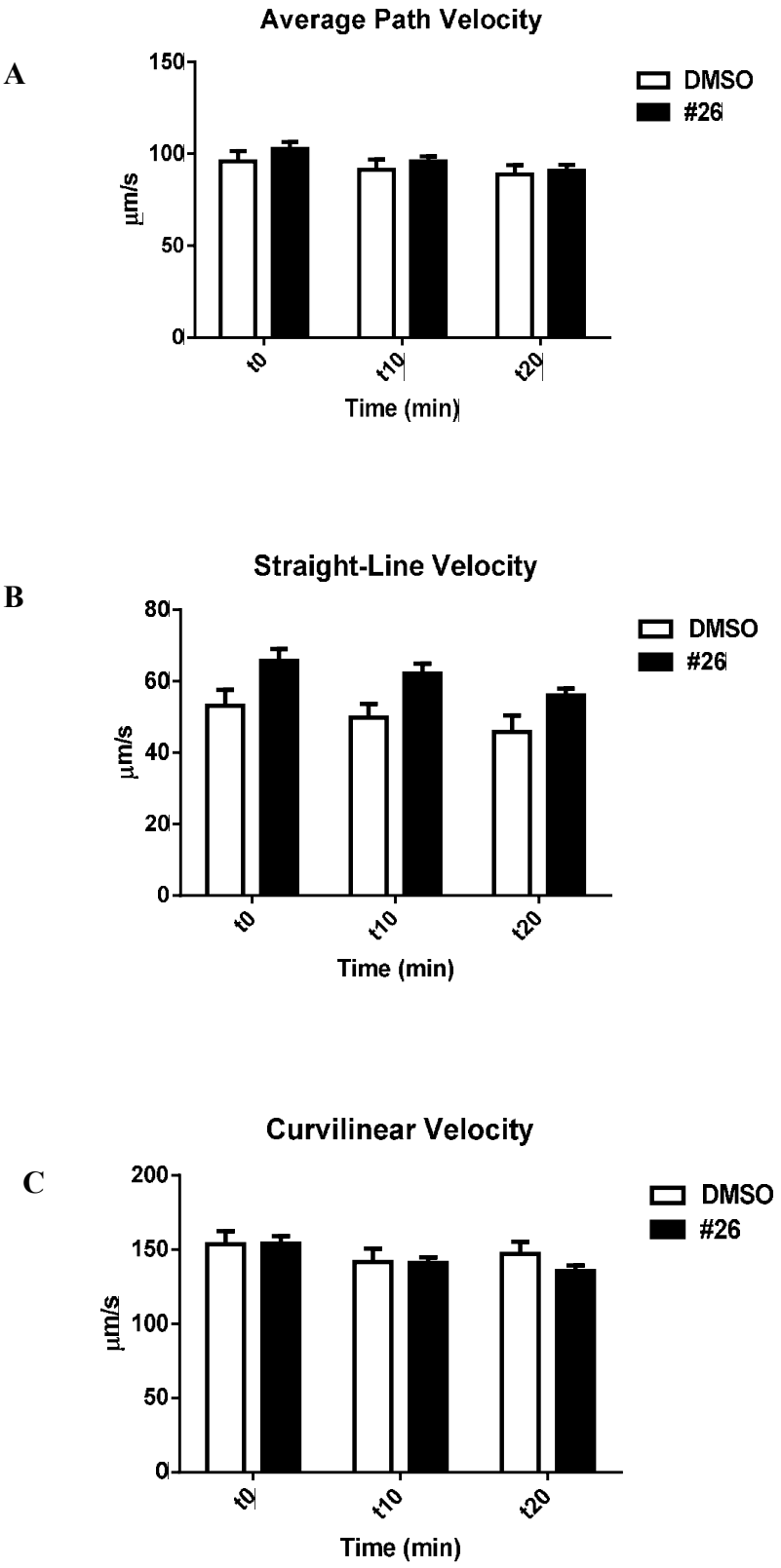


Figure 5-4: Effects of compound (#26) on swimming velocities of boar spermatozoa. (A) Average path velocity (VAP). (B) Straight-line velocity (VSL). (C) Curvilinear velocity. (DMSO: 1% v/v) mean: ±SEM. Two Way ANOVA.

5.5 Discussion

Addition of phosphodiesterase inhibitors, such as caffeine and theophylline, during sperm-oocyte co-incubation is generally employed in many porcine IVF systems (Suzuki et al., 2005, Yoshioka et al., 2003, Abeydeera and Day, 1997a). These compounds are considered to inhibit the degradation of cyclic nucleotides, resulting in an increase in intracellular nucleotide, as well as affect various aspects of sperm capacitation and acrosome reaction. Addition of caffeine to boar spermatozoa during in vitro fertilization promotes sperm penetration; however, this is usually accompanied by an increase in polyspermic penetration (Abeydeera and Day, 1997b, Funahashi and Nagai, 2001). In this present study, it is demonstrated that compound #26 at 100μM could stimulate the ability of spermatozoa to penetrate in vitro-matured porcine oocytes without being accompanied by high rate of polyspermic penetration.

In this present study, the proportion of oocytes penetrated in the presence of compound #26 were low (39%), but incidence of monospermic penetration was high (96%). The low rate of oocyte penetration may be due to the concentration of sperm cells used (1x 10^5cells/ml). In many porcine IVF systems, sperm concentration ranging from 10^6-10^7cells/ml has been used during sperm to oocyte co-incubation and the mean number of penetrated oocyte tends to increase with increasing sperm concentration (Nagai et al., 1984, Funahashi and Day, 1997, Funahashi et al., 2000, Wang et al., 1991). However, presence of a large number of spermatozoa has been associated with high polyspermy rates (Funahashi and Nagai, 2001). Here, concentration of 1x 10^5cells/ml was used: 1) to ensure that addition of compound #26 during sperm-oocyte co-incubation is what potentiates oocyte penetration; 2) in order to rule out confounding effects relating to addition of large number of spermatozoa during sperm-oocyte co-incubation.

Use of optimal sperm-oocyte co-incubation time interval is crucial in porcine IVF systems in order to allow sufficient sperm-oocyte interaction and prevent high incidence of polyspermic penetration. Data presented here suggest that sperm-oocyte co-incubation interval of 3hrs, in the presence of compound #26, followed by post-incubation in fertilization media without sperm and compound, is efficient for sperm-oocyte interaction and promotion of high monospermic penetration rate. Gamete co-incubation of 16-21hrs has been reported (Martinez et al., 1996), however, the deleterious effect of reactive oxygen species (ROS) during prolonged gamete co-incubation has been extensively documented (Guérin et al., 2001). Although, ROS may originate from oocyte metabolism, as a result of enzymatic activity, it is plausible that prolonged interval of gamete co-incubation may result in an excess of dead sperm leading to generation of ROS in culture. A recent study demonstrate that ROS levels generated by spermatozoa significantly increases after 18 h in culture (Enkhmaa et al., 2009). Therefore, 3hrs gamete co-incubation time used in this study could potentially mean reduction in the level of ROS in culture media.

In most porcine IVF systems (Grupen, 2014, Abeydeera and Day, 1997a, Funahashi and Romar, 2004), caffeine is frequently used as an additives to sperm suspensions in order to improve sperm characteristics and stimulates induction of capacitation. Caffeine is generally assumed to work by inhibiting phosphodiesterase activity, resulting in elevation of complementary adenosine monophosphate levels in spermatozoa. Results presented here showed that there is no significant effect of compound #26 on sperm velocities (VAP, VSL and VCL) and the percentage of total motile spermatozoa over the time interval analysed (t0-t20). However, a significant increase in progressive motility was observed at t0, when the compound was added to sperm suspension, but not at t10 and t20 respectively. The lack of effect of compound #26 on boar sperm motility is particularly notable, since this differs from human studies (Chapter 3 and Chapter 4) in which addition of compound #26 leads to an

increase in the percentage of total and progressively motile sperm respectively. In mammalian cells, multiple PDE types and subtypes, including splice variant do exist and they differ in relation to their sensitivity to activators/inhibitors. Particularly in sperm cells, relatively little is known about PDE types/subtypes, their specificities/affinities and their cellular sub localisation/distributions. This result could highlight a possible species-specific difference in the effect of compound #26 with respect to human and boar sperm motility.

Since, in this present study, compound #26 was found not to have any significant effect on boar sperm motility; the observed increase in oocytes penetration rate may be due in part to enhancement of capacitation related events. Although effects of compound #26 in relation to capacitation related events was not analysed in this study, other studies have shown that addition of phosphodiesterase inhibitor or cell permeable cAMP analogue to boar sperm could induce a rapid and major change in the lipid architecture of sperm plasma membrane (Harrison et al., 1996). These surface changes are crucial because it enables sperm cells to bind to the extra cellular matrix of the oocyte (zona pellucida) during fertilization. Evidence has been provided over the years to show that, among other molecular changes, capacitation involves a transition in the plasma membrane from a stabilized to a destabilized state and only capacitated sperm cell can bind to the ZP, leading to fertilization of oocyte (Flesch and Gadella, 2000, Gadella et al., 2008). Alternatively, since compound #26 was added during gamete co-incubation, it is plausible that the compound is also exerting its effects on the oocyte; thereby leading to an enhanced penetration rate. While there are no studies analysing the effects of PDE inhibitors on oocytes during gamete co-incubation, previous studies have reported that addition of PDE inhibitors during in vitro maturation of oocytes improved nuclear maturation by regulating meiotic and cytoplasmic maturation (Nogueira et al., 2005, Rose et al., 2013, Gharibi et al., 2013, Huang et al., 2013).

In conclusion, the result presented in this chapter has shown that compound #26 has no significant effect on boar sperm motility and velocity characteristics analysed. However, addition of this compound in fertilization media during gamete co-incubation can promote penetration of in vitro matured porcine oocytes. The 3hr interval of gamete co-incubation was found to be efficient for sperm-oocyte interaction and a remarkable proportion of fertilized oocyte, using this short incubation time, resulted in monospermic fertilization. Nevertheless, further study is needed to determine if increasing sperm concentration and gamete co-incubation time will increase penetration rate. Additionally, although the primary aim of this chapter is to determine the effect of compound #26 on porcine IVF system, a direct comparison with caffeine is needed using exact protocols described in this chapter. Furthermore, the effect of the compound on capacitation related events of boar sperm, such as plasma membrane modification, needs to be characterised.

Chapter 6.

Optimisation and validation of HPLC technique for the quantification of cAMP in boar sperm

6.1 Introduction

Adenosine 3′, 5′-cyclic monophosphate (cAMP), is a ubiquitous intracellular second messenger that is involved in the regulation of numerous physiological functions in mammalian sperm cells (De Jonge, 2005, De Jonge et al., 1991, Visconti et al., 1995b, Visconti et al., 2002). Cyclic AMP stimulates activation of protein kinase A (PKA) which in turn, by indirect action through SRC (Lawson et al., 2008, Baker et al., 2006), leads to the phosphorylation of tyrosine residues on other proteins. The central role of cAMP-PKA pathways in regulating numerous physiological functions in sperm has been established by many studies (Buffone et al., 2014, Liu et al., 2013, Buffone et al., 2009, Munire et al., 2004). Evidence from mouse models and human data – including those presented in Chapter 3 and Chapter 4 – is that cAMP-PKA pathway is involved in sperm capacitation, motility and hyperactivated motility and loss or diminished capacity of any of these processes is associated with infertility (Burton and McKnight, 2007). Consequently, based on its central role, the determination of cAMP levels in mammalian sperm cells has great practical importance for pharmaceutical and biomedical research.

However, measurement of cyclic nucleotides from sperm cells is often a challenging task, (characterised by low concentration, instability of nucleotides, post-extraction hydrolysis), thus, experimental limitations have impeded our ability to routinely and efficiently detect cyclic nucleotide in mammalian sperm. Currently, there are few /no studies examining the intracellular dynamics of a wide range of molecules in mammalian sperm; in order to generate a biochemical profile of the effects that physiological processes and pharmacological agents have on sperm cells. Additionally, the two main methods currently in vogue for the detection of cyclic nucleotides in mammalian sperm cells are expensive, labour intensive, have low detection levels/range, as in the case of enzyme-linked immunosorbent

assay (ELISA), and involve the use of radio activity (radio immunoassay). In this regard, a highly sensitive and selective method is required for routine quantification of cyclic nucleotides changes in mammalian sperm.

For non-germinal cell types, determination of cyclic nucleotide pool by high performance liquid chromatography is a well-established procedure (Lorenzetti et al., 2007, Ohba et al., 2001, Prado et al., 2013). HPLC is an analytical technique based on the separation of molecules due to differences in their structure and/or composition (Malviya et al., 2010, Dahimiwal et al., 2013). In HPLC, separation is accomplished by injection of a small amount of dissolved sample into a moving stream of liquid (called the mobile phase) that passes through a column packed with particles of stationary phase. Separation of a mixture into its components depends on the differences in specific chemical or physical interactions of each component with the column. In many analytical chromatography procedures involving nucleotide analysis, ion-pairing reversed phase HPLC (RP-HPLC) is often a method of choice due to its ability to provide stable and reproducible results. Additionally, a combination of UV and fluorescence detection mechanism enhances the detection of low concentration cyclic nucleotides. This method of separating components of miscible solute in solution will be innovative and instructive for the assessment of sperm biochemical dynamics; therefore needs validation and standardisation.

6.2 Aims and Experimental Design

In the present work, an isocratic HPLC method with ion–pair system was optimised and validated for the determination of cellular nucleotide (cAMP) concentrations in extracts from

boar sperm cells. Boar sperm was used as a surrogate model to human sperm due to cost and ease of availability of sperm sample. Additionally, the effect of phosphodiesterase inhibitors on the level of cAMP in boar sperm was examined. On the basis of results from previous chapters, it is hypothesised that PDEi compounds will enhance cAMP levels in boar sperm cells.

6.3 Materials and Methods

6.3.1 Chemicals and Reagents

Adenosine 3′, 5′-cyclic monophosphate (cAMP) sodium salt, KH_2PO_4 and HPLC grade: acetonitrile, methanol and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich, UK. The deionised water used for standard and mobile phase preparation and dilution were from a Millipore water purification system.

6.3.2 Capacitating Conditions

Capacitating media (CM) was composed of 5mM KCl, 1mM KH_2PO_4, 95mM NaCl, 5.55mM glucose, 25mM $NaHCO_3$, 2mM $CaCl_2$, 0.4% BSA and 2.5mM pyruvate (pH 7.4). Non-capacitating media (NCM) lacked calcium, bicarbonate and BSA and consisted of 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, 137mM NaCl, 5.55mM glucose and 2.5mM pyruvate (pH 7.4). $3x10^7$ cells were pelleted and resuspended in 1ml of either CM or NCM and incubated at 39°C for 3hours. Cells in CM were kept in CO_2 incubator with the lids off while those in NCM with lids closed.

6.3.3 Preparation of Standard Solution

Adenosine 3′,5′-cyclic monophosphate (cAMP) sodium salt (Molecular Weight: 369.20g/mol) standard was prepared by dissolving precisely weighed 1mg of cAMP-salt(HPLC grade (≥98.5%) in 10mL of deionised water in volumetric flask and this was then serially diluted (x5) in deionised water (See below). All calibration procedures and experiments were performed using freshly made standards.

Table VIII: cAMP concentration and serial dilutions of cAMP standard solution

Serial Dilution No	Amount of cAMP (g/10ml)	Initial No of Moles	Conc. of Solution (M)	Injection Volume (µl)	Amount of cAMP per Injection (g)	Final No of Moles
1	$1x10^{-3}$	$2.71x10^{-6}$	$2.7x10^{-4}$	50µl	$5x10^{-6}$	$1.35x10^{-8}$
2	$1x10^{-4}$	$2.71x10^{-7}$	$2.7x10^{-5}$	50µl	$5x10^{-7}$	$1.35x10^{-9}$
3	$1x10^{-5}$	$2.71x10^{-8}$	$2.7x10^{-6}$	50µl	$5x10^{-8}$	$1.35x10^{-10}$
4	$1x10^{-6}$	$2.71x10^{-9}$	$2.7x10^{-7}$	50µl	$5x10^{-9}$	$1.35x10^{-11}$
5	$1x10^{-7}$	$2.71x10^{-10}$	$2.7x10^{-8}$	50µl	$5x10^{-10}$	$1.35x10^{-12}$
6	$1x10^{-8}$	$2.71x10^{-11}$	$2.7x10^{-9}$	50µl	$5x10^{-11}$	$1.35x10^{-13}$

n= moles of pure substance. m= mass of substance in grams. M= Molar mass in g/mL. L= Litres

$$n = \frac{m}{M}, \quad \text{Molarity} = \frac{moles}{L}$$

For Serial dilution No 1:

$$n = \frac{1mg}{369.2g/ml} = 2.71x10^{-6} \text{ moles in}$$

10ml (or 10000µl)

Concentration of Solution: $\frac{2.71\times10^{-6} \text{ moles}}{0.01L} = 2.7x10^{-4}$ M

Number of mole in 50 µl injected volume: $\frac{2.71\times10^{-6} \text{ moles} \times 50\mu l}{10000\mu l} = 1.35x10^{-8}$ moles or 13500 pmoles

6.3.4 Chromatographic Equipment and Conditions

The HPLC system (see Figure 6-1) consists of Waters: 1525 Binary HPLC pump, 2487 Dual λ Absorbance Detector, 2475 Multi λ Fluorescence Detector and 717 plus Autosampler. The chromatographic separation was accomplished on a Synergi™ 4µm Fusion – RP80A (Phenomenex, Cheshire, UK) C18 analytical column (150mm× 4.6mm Internal Diameter (I.D), 4µm particle size) Column temperature: ambient temperature; Flow rate: 1.0 ml/min; Injection volume: 50 µl. The detection wavelength for cyclic nucleotide was set at 260 nm and the system was controlled and data analyses were performed on Water's Breeze Version 3.20 software.

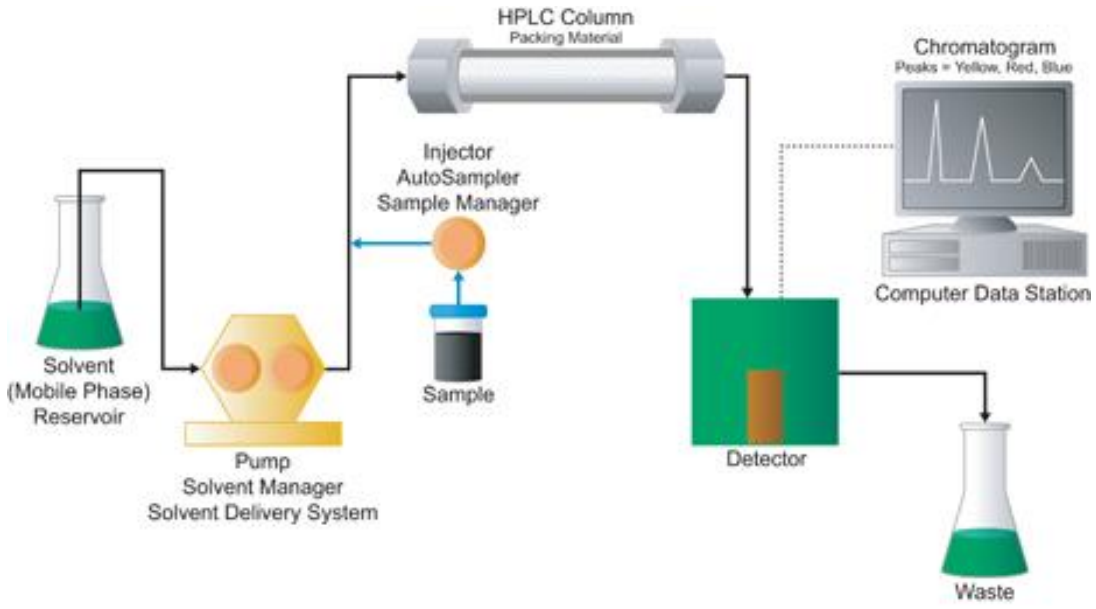


Figure 6-1 Diagram of the general structure of a HPLC system

6.3.5 Mobile Phase

The mobile phase consisted of Buffer **A**, 20 mM KH_2PO_4, pH 2.5. This is prepared by dissolving 2.7218g of HPLC grade KH_2PO_4 in ultrapure water (in a volumetric flask) and pH adjusted with 2.5% phosphoric acid solution (Final volume of buffer: 1L). Buffer **B**, is ultrapure water supplemented with 0.1% TFA and 0.1% acetonitrile (ACN) and Buffer **C**, 20mM Phosphate buffer supplemented with 0.1%TFA and 0.1%ACN (final pH 2.08). All buffers used were filtered through a 0.22 μm Millipore membrane filter before use.

6.3.6 Sperm Preparation and Treatment

Boar sperm, (Pig Improvement Company UK Limited) stored up to 2 day at 16-17^{o}C in TRIXCell extender (IMV technologies), was pelleted by centrifugation (15 min, 1500 rpm). The sperm pellet was resuspended in CM and incubated for 3hrs under capacitating conditions (39^{o}C and 5% CO_2 humidified atmosphere) after sperm concentration was determined by haemocytometer count (Sperm concentration: $3x10^7$/ml). After incubation, sperm cells were exposed to compounds (PDEi) for 20min before extraction of cytosolic content of the cells.

6.3.7 Cell Extraction of Cyclic Nucleotides

For the extraction of cyclic nucleotides, two alternative techniques were attempted; methanol extraction and sonication. Methanol is routinely used during cells extraction (Rose and Oklander, 1965, Ferrer et al., 2011) and sonication (Ijiri et al., 2011, Han et al., 2011) involves mechanical disruption of cells without introduction of other compounds in the cell extract.

6.3.7.1 Methanol

After incubation and treatment (see above), cell pellet ($3x10^7$/ml) was formed by centrifugation (15 min, 1500 rpm) and cells were lysed, after removing supernatant, by resuspending in 500µL ice-cold 100% HPLC grade methanol (-20^{o}C, 10min). To separate the particulate and soluble fractions, lysed samples were centrifuged (30 min, 3000 rpm), the supernatant was taken and vacuum centrifuged (~1.5hr at 60^{o}C; Eppendorf Concentrator 5301) to evaporate the methanol and the lyophilised soluble fractions was resuspended in 500µL of Buffer A. This is then transferred into labelled vials and loaded onto the HPLC system.

6.3.7.2 Sonication

After incubation and treatment (see above), cell pellet was formed by centrifugation (15 min, 1500 rpm) and after removing supernatant, cells were resuspended in 500µL Buffer **C** (see above). Mechanical disruption of cell membrane was achieved by sonication using a Micro tip (Sonics & Materials Model: V1A). Sonication experiments were conducted at 10 s, 15s, 20s, 25s and 30s at 95% power and amplitude of 4 (samples were kept on ice at all time). After sonication, the particulate and soluble fractions were separated by centrifugation (15 min, 1500 rpm at 4^{o}C; Eppendorf Centrifuge 5417R). The soluble fractions were then transferred into labelled vials and loaded onto the HPLC system.

6.3.8 Derivatization of cAMP Standards and Cell Extract

In liquid chromatography analysis, fluorescent derivatives; such as UV chromophores and fluorophores, are often introduced into sample to increase their sensitivity to UV absorption and fluorescence detection. For the conversion of nucleotides into fluorescent derivative,

500µl of freshly prepared cAMP standard samples and 500µl of cell extract (lyophilised soluble fraction in Buffer A) were mixed with 50µl of chloroacetaldehyde ($ClCH_2CHO$) and 500µl of 2.5% phosphoric acid in capped 1.5ml Eppendorf tubes. These mixtures were vortex mixed and kept at 80ºC for 30 minutes. After the reaction (Figure 6-2), the tubes were immediately placed in a 4°C refrigerator to stop the reaction (Zhang et al., 2006). These solutions were transferred into labelled vials and loaded onto the HPLC system.

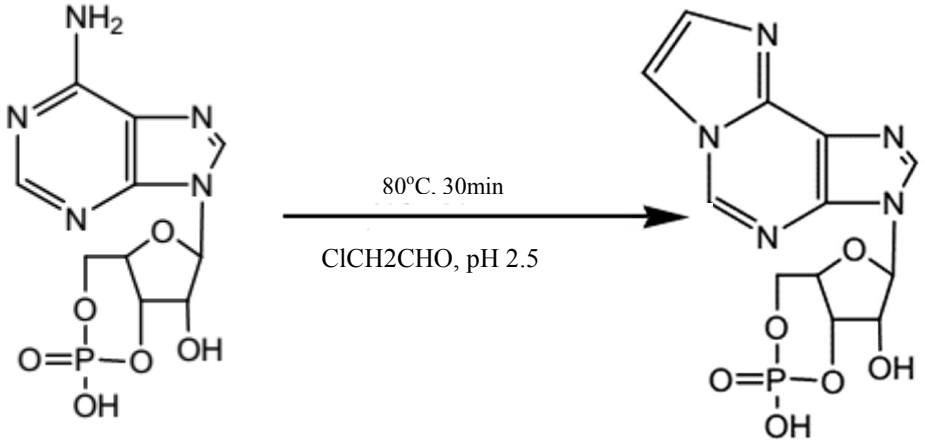


Figure 6-2: Derivatization reaction of cAMP by 2-chloroacetaldehyde

6.3.9 Assay Validation and Statistical Analysis

The analytical method was validated by evaluating linearity through the calibration curve obtained with six concentration levels of cAMP standard solutions. By analysing the linear regression of the peak-height versus concentration, the determination coefficient, the intercept and the slope were calculated. The limits of quantification (LOQ) and limits of

detection (LOD) was determined based on the standard deviation of the response and the slope of the calibration curve as recommended by ICH guidelines (Guideline, 1997).

$$\mathrm{LOQ} = \frac{10\sigma}{\mathrm{S}} \ , \ \mathrm{LOD} = \frac{3.3\sigma}{S}$$

Where σ = the standard deviation of the response

S = the slope of the calibration curve

Statistical analysis of the effects of different compound treatment conditions and sonication time on the level of cAMP in boar sperm was conducted using analysis of variance (ANOVA). The statistical analysis was carried out using GraphPad Prism 6 (GraphPad Software, California, USA). Differences were considered significant at a $P < 0.05$.

6.4 Results

6.4.1 Effect of Mobile Phase on Retention Time of Standard

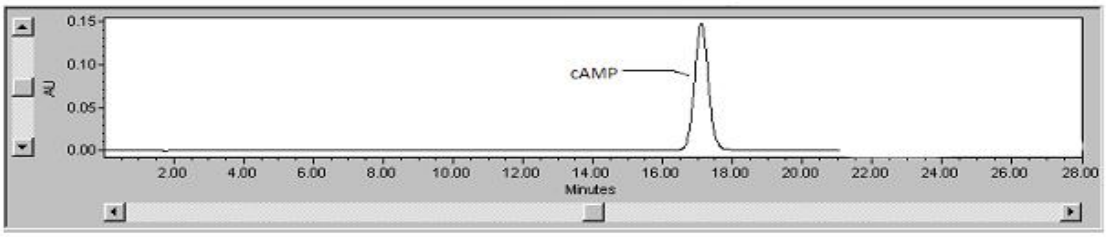
The separation of organic compounds in reverse phase chromatography is due to the different binding properties of the solutes present in the sample as a result of the differences in their hydrophobic properties. Thus, by manipulating the hydrophobic properties of the mobile phase, through the addition of organic modifiers to lower the polarity of the solution, separation of solutes can be fine-tuned and the retention time (the time between injection and detection) modified. Here, the effect of three mobile phases on retention time of cAMP standard was assessed.

Figure 6-3A showed that by using Buffer **A**, 20mM phosphate buffer (pH 2.5), the retention time of cAMP standards was at ~17 min. A linear calibration curve is demonstrated in Figure 6-3B and this showed that for Buffer A, the lower limits of quantification (LOQ) of cAMP,

that is, the lowest concentration of cAMP that can be reliably detected, is at $1.494x10^{-6}$M (0.55µg/mL) with $r^2 = 0.9707$ (Figure 6-3B). With Buffer **B**, ultrapure water supplemented with 0.1% TFA (ion-pairing agent) and 0.1% ACN (organic solvent) (pH 2.2), the retention time of cAMP standards was reduced to ~11min (Figure 6-4A), however, the limit of quantification (LOQ) while using this buffer was at $1.27x10^{-6}$M (0.46 µg/mL) with $r^2 = 1$ (Figure 6-4 B). The retention time of cAMP standards when using Buffer **C**, 20mM Phosphate buffer supplemented with 0.1%TFA and 0.1%ACN (pH 2.08), was at ~ 7min (Figure 6-5A) and the limit of quantification of cAMP is at $1.56x10^{-6}$M (0.57µg/mL) with r^2 =0.996 (Figure 6-5B); and there is no significant difference between the LOQ of the three buffers (See APPENDIX 2 for calculation of LOQ). For the three buffers, the lower limit of detection (LOD), that is, the lowest concentration of cAMP that can be reliably distinguished from blank or background noise is: Buffer A: 4.930×10^{-7}M (0.18 µg/mL); Buffer B: 4.199×10^{-7} M (0.15 µg/mL) and Buffer C: 5.153×10^{-7} M (0.19 µg/mL) respectively.

For each mobile phase, the method of cAMP separation and the precision of the system showed good repeatability and reproducibility. The percentage of relative standard deviation (% R.S.D), which is a measure of precision and repeatability of an assay in analytical chemistry, was calculated from the peak area ratios for each concentration of cAMP standard injected. The % R.S.D is similar for the three mobile phase tested; and for each concentration of standard injected; it was within 2%.

A



B

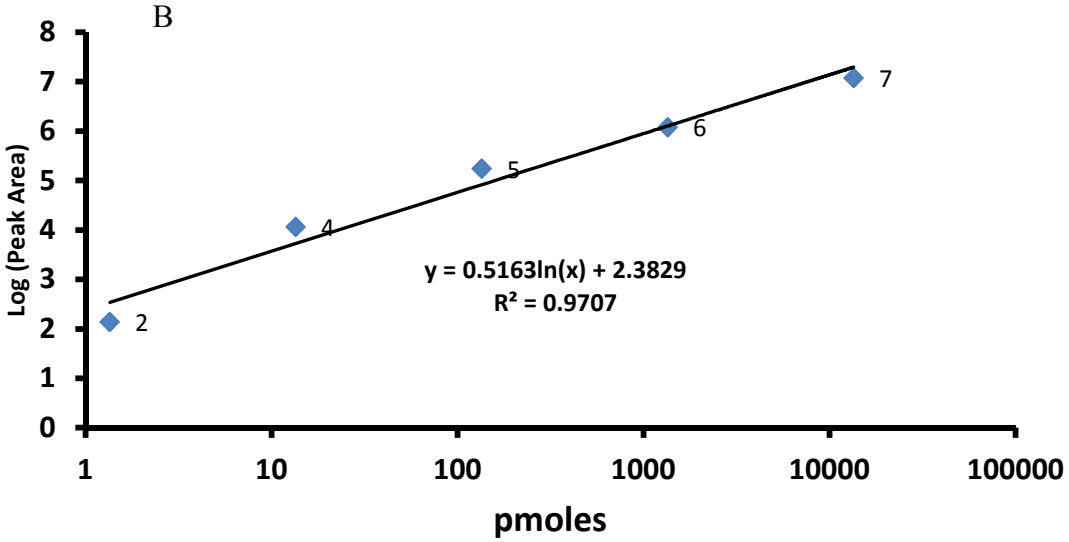
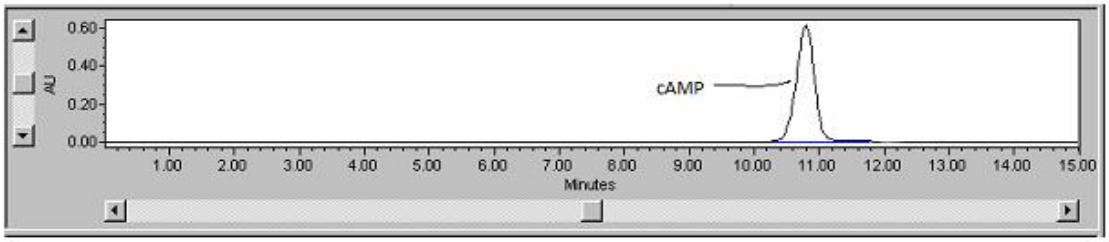


Figure 6-3: Effect of Buffer A (20mM Phosphate buffer) on retention time of cAMP standard. (A) Chromatogram of cAMP standard peak. 1.35×10^{-8} to 1.35×10^{-13} moles of cAMP standard prepared in ultrapure water were injected into HPLC. The retention time was ~ 17min. (B) Linear relationship of cAMP concentration vs. UV peak area. Area under the curve of peaks were normalized by taking Log_{10} and plotted versus given cAMP concentrations. (n=3, r^2 = 0.9707).

Mobile phase	20 mM phosphate buffer solution (pH 2.5)
Flow rate	1.0 mL/minute
Temperature	25 °C
Detection	260 nm
Retention time	~17min

A



B

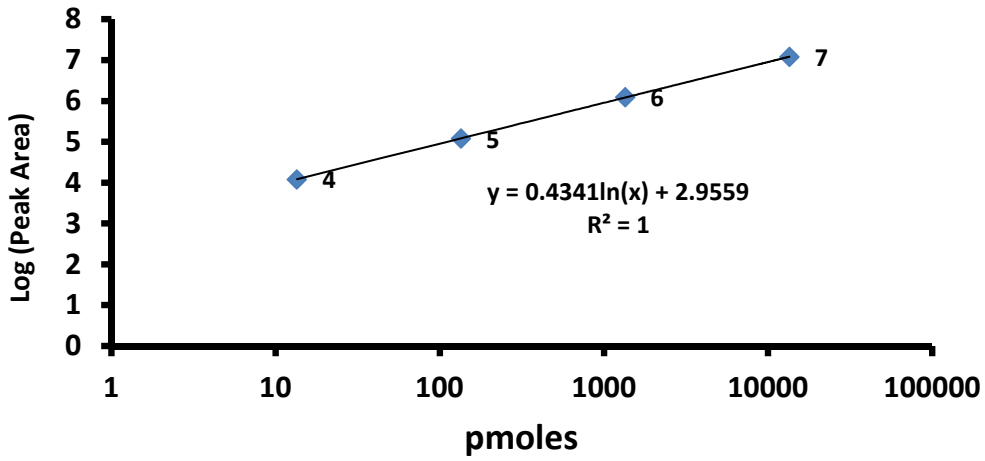
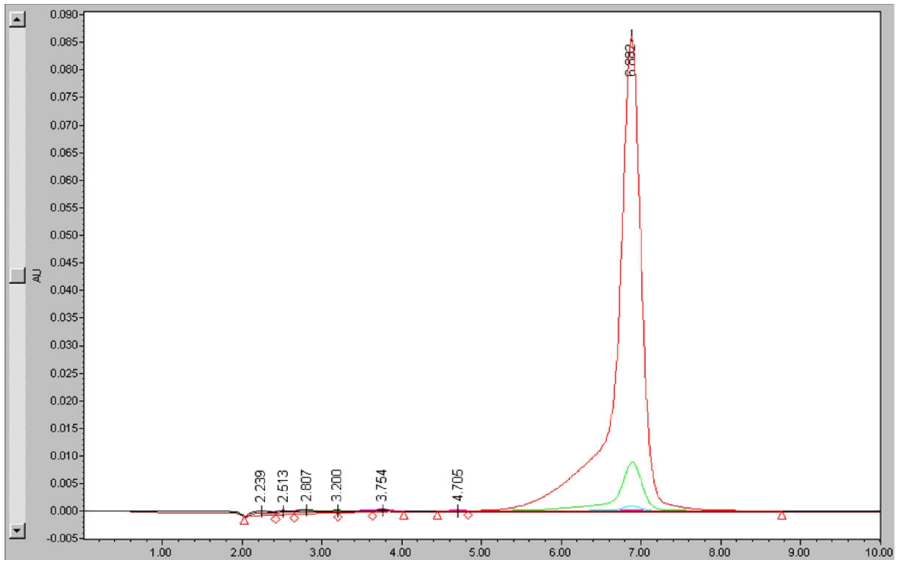


Figure 6-4: Effect of Buffer B (Ultrapure water supplemented with 0.1% TFA and 0.1% (ACN)) on retention time of cAMP standard. (A) Chromatogram of cAMP standard peak. 1.35×10^{-8} to 1.35×10^{-13} moles of cAMP standard prepared in ultrapure water were injected into HPLC. The retention time was ~ 11min. (B) Linear relationship of cAMP concentration vs. UV peak area. Area under the curve of peaks were normalized by taking Log_{10} and plotted versus given cAMP concentrations. (n=3, $r^2 = 1$).

Mobile phase	Ultrapure water supplemented with 0.1% TFA and 0.1% acetonitrile (ACN)
Flow rate	1.0 mL/minute
Temperature	25 °C
Detection	260 nm
Retention time	~11min

A



B

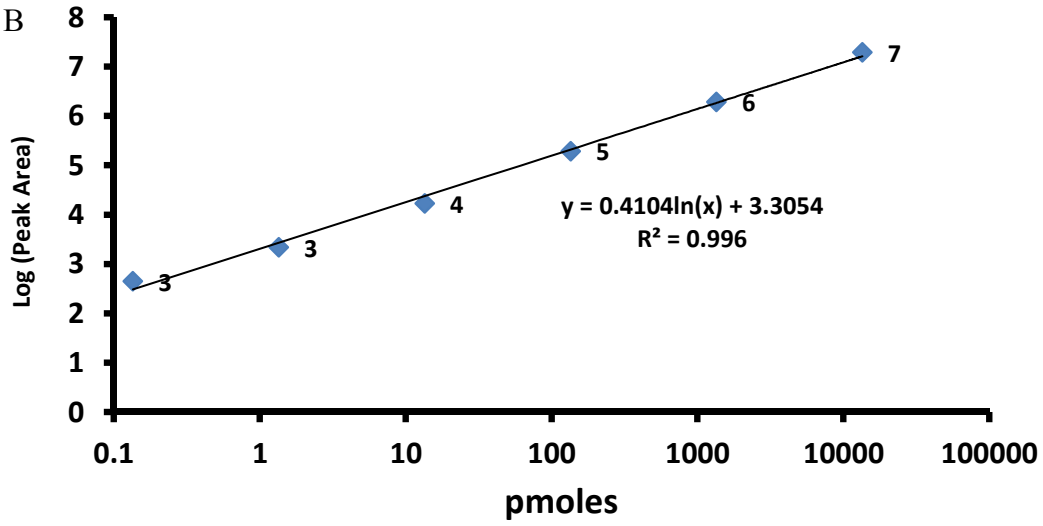


Figure 6-5: Effects of Buffer C (20mM Phosphate buffer supplemented with 0.1%TFA and 0.1%ACN) on retention time of cAMP standard. (A) Chromatogram of cAMP standard peak. 1.35×10^{-8} to 1.35×10^{-13} moles of cAMP standard prepared in ultrapure water were injected into HPLC. The retention time was ~ 7min. (B) Linear relationship of cAMP concentration vs. UV peak area. Area under the curve of peaks were normalized by taking Log_{10} and plotted versus given cAMP concentrations. (n=3, $r^2 = 0.996$).

Mobile phase	20mM Phosphate buffer + 0.1%TFA+0.1%ACN
Flow rate	1.0 mL/minute
Temperature	25 °C
Detection	260 nm
Retention time	~7min

6.4.2 Effect of Cell Extraction on Cyclic Nucleotide Detection by HPLC

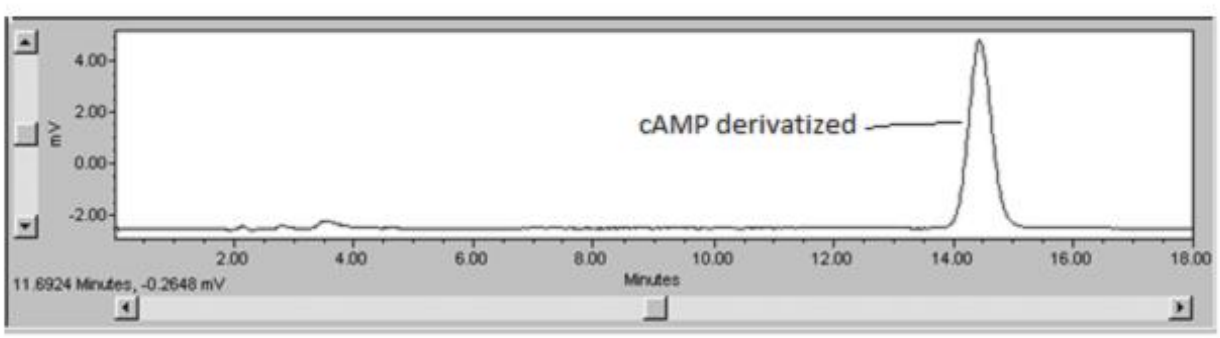
Two approaches were adopted for the extraction of cAMP from boar sperm cells. The initial approach involves the use of 100% methanol (-20^{o}C for 10min) to lyse cell pellet of capacitated boar sperm. The sample is then centrifuged to separate membrane and cytosolic supernatant. The supernatant is vacuum centrifuged to evaporate the methanol and the lyophylised cytosolic pellet is resuspended in 500µl of Buffer A. However, when this sample was run on the HPLC system, using the exact same conditions used for the detection of cAMP standards in Buffer A (Figure 6-3), no peak was identified (n=3) at the retention time seen in Figure 6-3. In order to improve sensitivity and quantification of cAMP in cell extract, both sample and cAMP standard solutions were derivatized and detected using a fluorescence detector. Figure 6-5A showed a chromatogram with distinct peak of derivatized cAMP standard, with retention time at ~14min. However, in derivatized cell extract, no peak similar to those found with derivatized cAMP standard could be identified (n=3).

The second approach uses sonication technique to lyse boar sperm cells. With this approach, cell pellet of capacitated boar sperm were resuspended in 500µl Buffer C, while keeping sample tubes on ice, cells were sonicated at various times (10s 15s, 20s, 25s and 30sec). Since sperm cells were resuspended in Buffer C, the exact same condition used for the detection of cAMP standards in Buffer C was used on the HPLC system. Using this approach cAMP extract from boar sperm cells can be quantified.

Figure 6-7, showed the peak area of cAMP from boar sperm cells. Using the linear regression equation, that is y= 0.4421ln(x) + 2.8593 the mass of cAMP in boar sperm for each sonication time was calculated (10s: 5.75 ± 1.15µg; 15s: 4.68 ± 0.95µg; 20s: 4.59 ± 0.84µg; 25s: 4.19 ± 1.07µg and 30s: 4.85 ± 0.61µg) and there is no significant difference in peak area when various sonication times were compared p= 0.7965 (n=4). To ensure

sonication does not lead to degradation of cAMP during cell lysis, solutions of cAMP standards were sonicated, for 30s each, using the exact same conditions used for cell lysis. Figure 6-8A and B showed that exposure of the cAMP standards to 30s sonication time does not leads to degradation of cyclic nucleotides or alter the sensitivity of the system, moreover, this data is comparable to non-sonicated sample in Figure 6-5B.

A



B

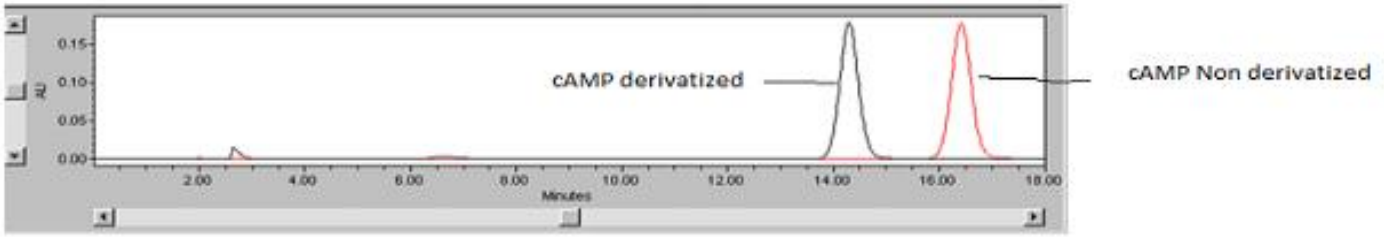


Figure 6-6: (A) Chromatogram of a derivatized cAMP standard. (B) A comparative view of derivatized and non derivatized cAMP standards.

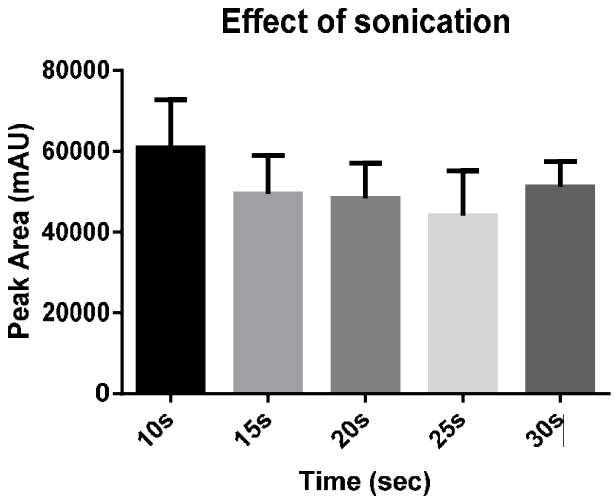
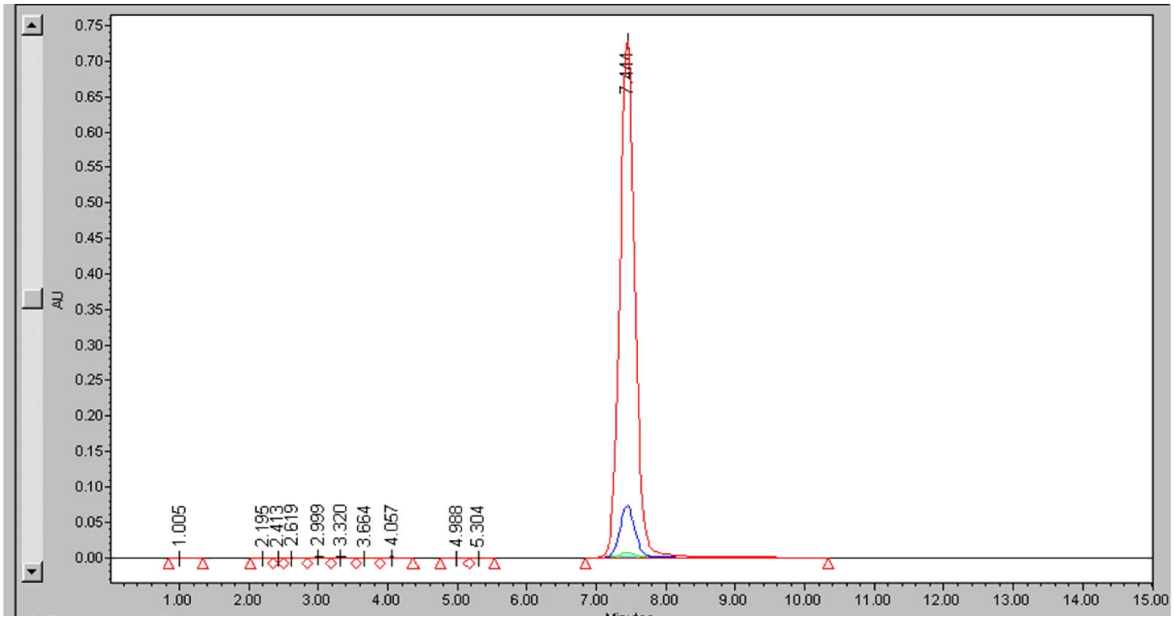


Figure 6-7: Effect of various sonication times on cyclic nucleotide detection on HPLC. Pellet of capacitated boar sperm cells were re-suspended in buffer C and cell lysis was achieved through sonication. There is no significant difference in peak area between sonication times. Peak area of cyclic nucleotides was determined by HPLC absorbance detector ±SEM n=4 One Way ANOVA.

A



B

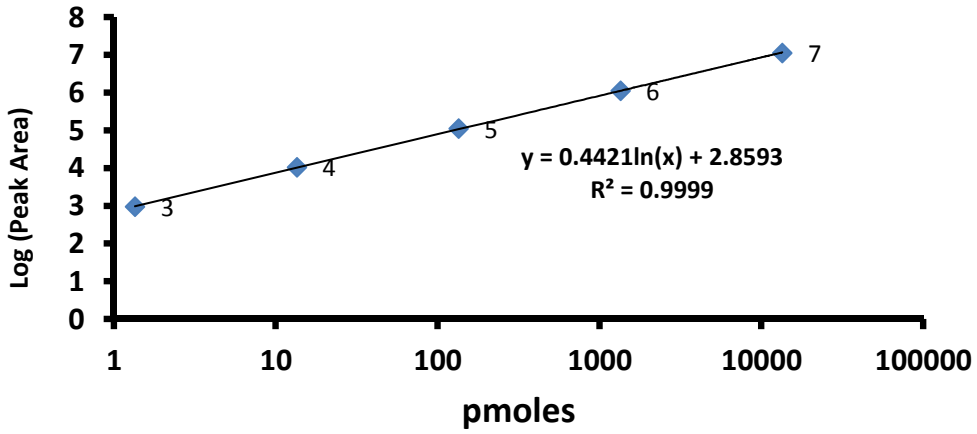


Figure 6-8: Effect of 30s sonication on solutions of cAMP standards. (A) Chromatogram of cAMP standard peak. 1.35×10^{-8} to 1.35×10^{-13} moles of cAMP standard were injected into HPLC using Buffer **C** as eluent**.** The retention time was ~ 7min. (B) Linear relationship of cAMP concentration vs. UV peak area. Area under the curve of peaks were normalized by taking Log10 and plotted versus given cAMP concentrations. (n=4, $r^2 = 0.9999$).

Mobile phase	20mM Phosphate buffer + 0.1%TFA+0.1%ACN
Flow rate	1.0 mL/minute
Temperature	25 °C
Detection	260 nm
Retention time	~7min

6.4.3 Effects of Phosphodiesterase Inhibitor on cAMP Level in Boar Sperm

To assess the effects of phosphodiesterase inhibitors (PDEi) on the level of cAMP, capacitated and non-capacitated boar sperm cells were exposed to PDEi (No drug (Control), #26, #36, #37, #38, IBMX, and DMSO (vehicle control)) for 20min. Figure 6-9A and B showed that there is no statistically significant difference in peak area when each treatment conditions were compared with each other. Thus, this data suggests that there is no significant difference in in the level of cAMP between treated and non-treated cells; both in capacitating and non-capacitating conditions.

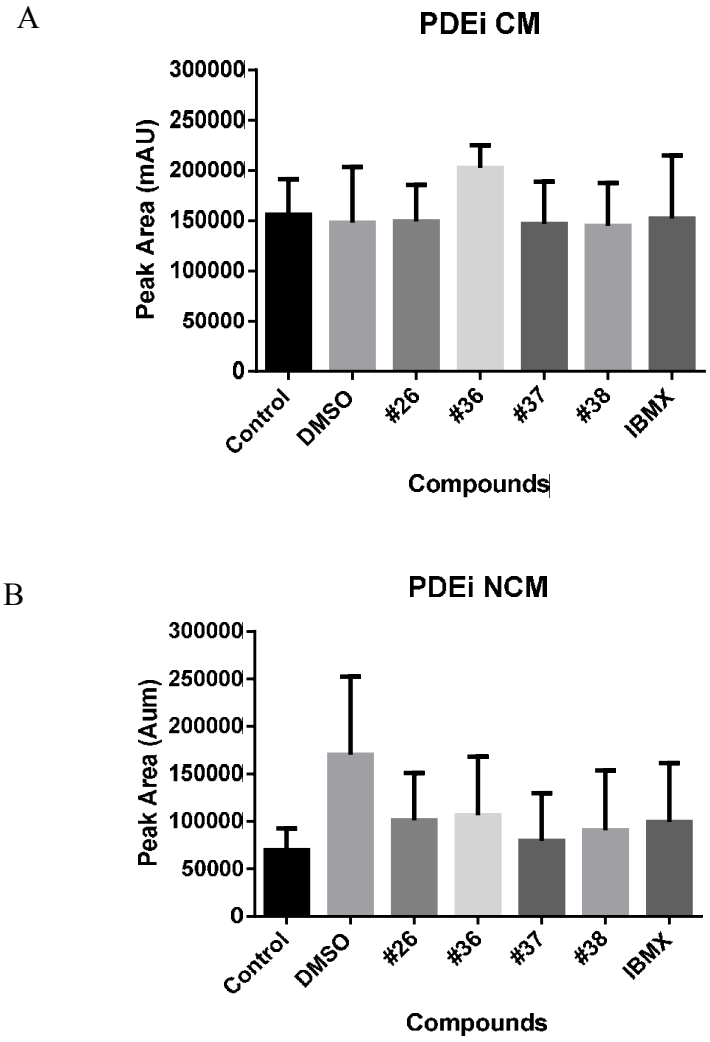


Figure 6-9: The effect of PDE inhibitors on cAMP level in boar sperm. (A) Capacitated and (B) non-capacitated boar sperm were exposed to PDEi for 20min. Cells were lysed by 30s sonication. Mobile phase buffer **C**. Peak area of cyclic nucleotides was determined by HPLC absorbance detector ±SEM n=3 One Way ANOVA

6.5 Discussion

Adenosine 3´, 5´-cyclic monophosphate (cAMP), is an important molecule that is involved in the regulation of numerous physiological functions in mammalian sperm cells (De Jonge, 2005, De Jonge et al., 1991, Visconti et al., 1995b, Visconti et al., 2002). Inside the cell, cAMP activates PKA which then leads to phosphorylation of tyrosine residues on other proteins by an indirect action (Lawson et al., 2008, Baker et al., 2006) and this signalling pathway has been shown to be important for the process of sperm capacitation, motility and hyperactivated motility (Buffone et al., 2014, Liu et al., 2013, Buffone et al., 2009, Munire et al., 2004). However, in sperm cells, detection of cyclic nucleotides has proved difficult because the two main methods are expensive, labour intensive, low throughput, can only detect one subtype of nucleotide at a time and have either a very limited detection range, as in the case of enzyme-linked immunoabsorbant assay (ELISA), or involve the use of radio activity (radio immunoassay). With advancement in liquid chromatography technique, use of HPLC technique provides an alternative technology to classical ELISA assays and this technique is suitable for high throughput automated detection systems.

In this study, HPLC technique was optimised and validated for the detection of cAMP. Using this technique, cAMP has been identified / quantified in boar sperm for the first time (Harrison, 2004, Harrison et al., 1996, Gadella and Harrison, 2002). The initial analysis involves determination of the mobile phase buffer that provides optimal sensitivity and selectivity and the quickest retention time for cAMP in solution. Previous studies have shown that mobile phase pH and the concentration of organic modifiers are some of the most important factors that affect the retention of ionisable solutes (Tanaka et al., 1978, Al-Hamdi et al., 2006, Shibue et al., 2005, Heyrman and Henry, 1999, Zou et al., 1991, Zisi et al., 2013). In this study, three mobile phase buffers (Buffer A, B and C) were tested to determine

their effects on cAMP retention. The concentration of the buffer (20 mM Phosphate) was chosen on the basis of column type as recommended by the manufacturer. While for each buffer there is high level of reproducibility regarding cAMP separation, the quickest total retention times, however, were associated with Buffer **C:** at ~ 7min followed by Buffer **B** (~11mim) and Buffer **A** (~17min) respectively. An explanation for this is that at increasing acidic pH, the basic groups of cAMP are protonated which results in an increase in polarity, thus the interaction between solutes and the stationary phase is altered, leading to decrease in retention time. Additionally, there is no significant difference between the limit of quantifications (LOQ) and detections (LOD) for all three buffers used; therefore, Buffer **C** was selected for the remainder of this study as it offered the quickest retention time for cAMP detection.

In this study, 100% methanol was used as the extraction agent to extract the intracellular content of boar sperm as methanol is frequently used for the extraction of cellular content during ELISA. Here, methanol extract was evaporated to dryness and reconstituted in Buffer A. However, the reconstituted methanol extract from boar sperm cells gave no chromatogram when analysed on the HPLC system. The experimental conditions were scrutinized and several eventualities that might explain this unexpected failure to detect cAMP in methanol extract were entertained. The initial consideration was that the concentration of cAMP in lyophilised extract was too low for UV detection; therefore, in order to increase sensitivity, the samples were derivatized. While derivatized cAMP standards gave a chromatogram, on the contrary, no cAMP peak was detected from lyophilised methanol extract of boar sperm. The effect of temperature (60^{o}C) to possibly cause degradation of cAMP during the process of lyophilising the methanol extract sample was also considered, however, cAMP standards incubated at 80^{o}C during the process of derivatization gave a chromatogram which is comparable to underivatized sample; suggesting no degradation as a result of high

temperature. While it was concluded that cAMP from methanol extracts might be lost while the sample were being concentrated, the effect of temperature as a degradation factor was excluded as a possibility.

A shift in retention time of cAMP standard was observed in the derivatized cAMP standard solutions (Figure 6-6). This may be due to change in the structure of the compound (Figure 6-2); however, the quantification of cAMP does not change. Contrary to what was observed with methanol extraction, mechanical disruption of boar sperm membrane using sonication techniques led to detection of cAMP peak; therefore an improvement on methanol extraction. Although, there is no significant difference in cAMP peak between the sonication times tested, 30sec sonication time was chosen for optimum cell lysis. Also, 30sec sonication does not lead to cAMP degradation (Figure 6-8).

Several studies have shown that cAMP-dependent pathway plays a crucial role in regulating various sperm functional events associated with capacitation and motility in many species (Visconti et al., 1999c, Kalab et al., 1998, Galantino-Homer et al., 1997, Leclerc et al., 1996a). Additionally, the intracellular levels of cAMP, the second messenger that activated PKA, can be modulated by treatment with activators or inhibitors that targets the cAMP-dependent pathway. Here, boar sperm treatment conditions were set-up to mimic the conditions of treatment used in human sperm experiments (Chapter 3 and 4), that is, samples were exposed to the compounds for 20min then the effect of PDE inhibitor (PDEi), in this case on the levels of cAMP in boar sperm was assessed. For boar sperm under the experimental conditions described in this chapter, no significant difference was observed for the level of cAMP peak between PDEi treated and untreated boar sperms samples under capacitating and non-capacitating condition of treatment.

The lack of effect of these compounds on boar sperm cells, that is, to induce an increased level of cAMP is particularly notable and it does not match the initial hypothesis. One explanation for this might be that on the basis of incubation time used in this experiment (20min exposure to compound), the time window for detecting an increase or significant difference has been missed. Several studies, both in human (Battistone et al., 2013, Brenker et al., 2012) and boar sperm (Harrison and Miller, 2000) have reported a rapid increase in cAMP levels (raising within 1 min) which then rapidly falls back to control levels within 10-60min. cAMP efflux could be another mechanism that could explain why no difference was observed in cAMP levels between PDE treated and untreated samples (Salut et al., 2013, Rodriguez et al., 2011, Lin et al., 2007). While inhibition of PDE activity is one means of modulating cAMP level, regulation of cAMP homeostasis within the cell can also be achieved by efflux proteins. Indeed, while it has not yet been identified in boar sperm, high affinity efflux pump for cAMP (MRP4) has been identified in other cell types (Godinho and Costa-Jr, 2003, Sassi et al., 2012, Hofer and Lefkimmiatis, 2007) and recently in bovine sperm (Salut et al., 2013). Presence of such efflux pump in human and boar sperm may account for the rapid decrease in cAMP levels that is often reported in literature. Fundamentally, it will be interesting to quantify cAMP levels in extracellular media during boar sperm capacitation or when cells are exposed to PDE inhibitors. Alternatively, it could be that these sets of PDEi have no effect on boar sperm motility. As discussed in chapter 5, it is possible that they are not targeting the PDE subtypes found in boar sperm thus no increase in cAMP levels. Another explanation in relation to the effects of PDE inhibitor on human sperm is that these set of compounds also target CatSper in addition to activation of cAMP/PKA signalling pathway. Indeed, preliminary patch clamp data from our research group, using another PDE inhibitor (Trequinsin hydrochloride a PDE3 inhibitor), shows that PDE inhibitor can target and stimulates CatSper activation.

Finally, the method described here, for the detection of cAMP from boar sperm, is reproducible, rapid to perform, and has good linearity and precision. While the primary aim of this section of study was to optimise HPLC technique in order to determine cAMP concentration in boar sperm, this technique can possibly be adapted to simultaneously measure array of intracellular nucleotides. Being able to simultaneously measure array of nucleotides will give an insight into the dynamics of nucleotide metabolism during in vitro incubation of sperm cells or when exposed to different pharmacological agents. This study has provided proof of concept that this technique is sensitive enough to detect cAMP in mammalian sperm, therefore justifies future use of HPLC technique for high throughput screening of cyclic nucleotides in human spermatozoa.

Chapter 7.

General Discussion and Future Work

The aim of this thesis was to (1) examine the effect of phosphodiesterase inhibitors on spermatozoa in order to identify compounds that have clinically relevant enhancement of human sperm motility. (2) Identify the signalling pathway(s) involved in the motility enhancing effects of identified compounds by targeting modulator and mediator of cyclic nucleotides. (3) Develop an animal IVF model to assess effects of compound on fertilization and (4) Optimise HPLC techniques for routine detection of cyclic nucleotides in sperm cells.

7.1 Key findings

Sperm motility still remains one of the most important prerequisites for achieving fertilization and pregnancy. Under physiological conditions, in vivo, the sperm must travel through the female reproductive tract to the site of the ovulated oocyte before fertilization can occur. While the development and use of assisted reproductive technique has allowed many infertile couples to conceive, there is currently no clinically approved drug that can be added to spermatozoa in vitro or taken by a man in order to improve fertility. Although many substances have been proposed for the stimulation of human sperm motility (e.g. gonadotropin hormones FSH, and LH), however, advances in our knowledge means that targeting intracellular pathway(s) directly involved in sperm motility is the most attractive means of enhancing sperm motility.

With respect to Chapter 3, using a two-phased drug discovery screening approach, 43 compounds with reported phosphodiesterase inhibitor activity were systematically screened and from these; several compounds were found to have robust and effective stimulation of human sperm motility. Indeed, 6 compounds were found to have strong effect on poor motility samples; stimulating a significant increase in percentage of total and progressive motility both in capacitating and non-capacitating conditions respectively. Importantly, results presented in Chapter 3 showed that not only do these compounds stimulate and

enhance motility; their effects on progressive motility were sustained for a period of up to 180min, when compounds were co-incubated with sperm samples. The clinical use of the compounds would involve washing and effective removal of compounds prior to use. The data presented in this thesis showed that short (20min) exposure of sperm cells to compounds is still effective in enhancing both total and progressive motility even 300min after compounds were washed off. These findings are clinically relevant because they indicated that if these compounds were adopted for clinical use they could be added to sperm and then washed off, since their effects still persist after removal, before IVF insemination or intrauterine insemination (IUI). Indeed, data presented in this thesis showed that the enhancement of motility observed in poor motility samples can be replicated in patient samples. Particularly in ICSI patient samples, and with respect to compound #26, #37 and 38, ≥79% of samples showed a significant increase in total motility and progressive motility respectively. Furthermore, ~63% of IVF samples showed a significant increase in total motility, the majority of sperm cells from this sample group have better swimming velocity; characterised by higher proportion of treated cells having VCL ≥120μm/s than control samples.

Since sperm motility is still one of the main factors that determine the type of ART a patient is assigned (that is IUI for mild, IVF for moderate and ICSI for men with severe, sperm dysfunction (Barratt et al., 2011)), the findings from this thesis may have implications with respect to current clinical practice. Several studies have demonstrated a significant correlation between conception in vivo and the number/concentration of motile sperm and/or percentage motile cells in subfertile couples (reviewed in Tomlinson et al., 1999, 2013). In view of the above, that is, by boosting sperm motility of current crop of patient samples, hypothetically patients that are currently assigned for ICSI due to poor sperm motility could be assigned to IVF or IVF patients could be converted to IUI which is a cheaper option. From this

perspective, application of compounds; such as those identified in this study, to boost sperm motility could potentially increase the availability of fertility treatments to a global population, thus using IUI instead of IVF and making cost effective IUI available to a much wider patient population. Since many ART treatments are now commercially driven, the findings presented here could have profound economic benefits for private patients undergoing ART or policy makers that make recommendations on government funded patients.

The ideal compound that can be used clinically to enhance sperm motility will affect motility without any detrimental effects on other sperm functions and physiology. The potential negative effects of these compounds on human sperm physiology were examined. While there is still controversy regarding the timing of acrosome reaction during sperm-oocyte interaction, the acrosome reaction is a crucial step in the process of fertilization. Thus it is acknowledged that premature induction of the acrosome reaction by compounds would be a negative effect. In this study, the acrosome of human sperm incubated with compounds remained intact. Additionally, to assess the effects of the compounds on steps leading to fertilization, the Kremer penetration test was used to establish whether sperm are more able to swim through viscous media when incubated with compounds. Viscous media (methylcellulose) was used to mimic the viscous environment encountered in the cervix during the journey of the sperm to the oocyte in vivo. Results demonstrate that there is a significant increase in the number of sperm at a marked distance when sperm have been incubated with compounds; the reason for this could possibly be due to an increased average sperm swimming velocity when incubated with the compounds. Further to this, plasma membrane translocation of phosphatidylserine, which is an indication of apoptosis, was examined. Tests such as this are crucial to establish the clinical benefits or detriment of using these compounds during ART procedures. The results presented in this thesis demonstrate no

negative effects of the compounds on the level of phosphatidylserine externalisation. Pieced together, these results are very encouraging; since they show that compounds have positive effects on human sperm functions under the conditions of treatment. However, further studies are required to investigate the exact effects of compounds on sperm ion channels (such as CatSper) that are implicated in other fertilization processes.

Evidence from this thesis clearly shows that compounds positively affect human sperm motility, enhance other sperm functions and they have no detrimental effects. Thus it was hypothesised that compounds could potentially enhance in vitro fertilization rate. Although, current ethical approvals preclude the use of human oocytes, an animal IVF system was developed to test this hypothesis. Using boar sperm and porcine oocyte as a model, the effect of co-incubating boar sperm and porcine oocyte with Ibudilast (compound #26) on fertilization rate was determined. Ibudilast was found to significantly enhance IVF rate. In porcine IVF system, high polyspermic rate is often reported as a result of using stimulant (such as caffeine) to aid IVF rate. On the contrary, the data presented in this thesis showed that the addition of Ibudilast during sperm-oocyte co-incubation leads to a significantly high monospermic rate under the experimental conditions used. While, Ibudilast was the only compound tested, other compounds identified in this study need to be tested so as to determine their effects on IVF rates. Additionally, experiments to investigate the effects of these compounds on human oocytes are still necessary to establish safety and clinical effectiveness before they are considered for use at a clinical level.

In this thesis, the possible molecular signalling pathway mediating the motility stimulating effects of compounds were evaluated. In spite of the importance of sperm motility to

fertility in mammalian species, relatively little is known about the signalling pathways that regulate motility. While there is evidence to suggest that cAMP-PKA pathways are involved in overall sperm motility, their role in specific aspects such as initiation and maintenance of progressive motility, and hyperactivated motility are not well characterised (Visconti et al., 1997). Furthermore, the major downstream targets of PKA are yet to be fully characterised. The results presented in chapter 4 showed that the compounds were working predominately through the cAMP-PKA pathway. Incubation of sperm cells with sGC and PKG inhibitors have no inhibitory effect on sperm motility, however, incubation of cells in the presence of PKA inhibitor significantly reduce basal progressive motility and hyperactivation respectively. Moreover, the design of the experiment in this study enables the delineation of the role of PKA regarding initiation and maintenance of progressive motility and ultimately stimulation of hyperactivated motility. Results presented here shows that PKA is critical for basal progressive motility and stimulation of hyperactivated motility in human sperm.

Data presented in chapter 5 highlighted a possible species-specific difference in the effect of Ibudilast with respect to motility. Ibudilast, contrary to its motility enhancing effect on human sperm, seems not to affect boar sperm motility. Although in this study, boar sperm cells showed high sensitivity to microscope slides used for motility analysis by CASA, no significant improvement in motility was recorded when PDEi treated cells were analysed on agarose-coated microscope slides. This result could possibly mean Ibudilast is not affecting the PDEi targets in the tails of boar sperm; thus, this might highlight a difference in subtype of PDE present in human and boar sperm respectively. Alternatively, it could also mean that the compound is influencing capacitation related events in boar sperm.

Finally, an HPLC technique was optimised and validated for the detection of cyclic adenosine monophosphate (cAMP) in boar sperm. The use of HPLC provides a high throughput means of quantifying cAMP level in boar sperm extracts. Additionally, while the focus here was to detect cAMP, this technique can be used for the simultaneous detection of other nucleotides in cell extract, thus overcoming the limitations of single solute determination approach of ELISA technique.

7.2 Future Work

While the work carried out for this thesis has identified several compounds that have robust and effective stimulation of sperm motility however, data for IVF/ICSI patients were from one assessment. Thus further studies are required in order to establish the robustness of responses and to determine whether responses are repeatable. This can be achieved by setting up a clinical trial that will test the effects of compounds on a bigger pool of IVF and ICSI patient sample. Furthermore, more studies are necessary to evaluate the effects of compounds on sperm Ca^{2+} ions and associated signalling pathways. Numerous studies (Tamburrino et al., 2014) have highlighted the importance of Ca^{2+} ion on overall sperm function in relation to sperm motility and other fertilization processes. Thus, work would need to be expanded upon regarding the effects of compounds on CatSper ion channel and calcium influx through CatSper and Ca^{2+} store mobilisation. With advances in patch clamping electrophysiology and single cell calcium imagining, these techniques can be used to explore the effects of PDE-I on sperm intracellular calcium ions and calcium source. Similarly, with regards to motility, the involvement of calcium regulated downstream pathways needs to be explored further. It was postulated in this thesis that Calmodulin-CaMKII pathway might be involved in supporting progressive motility of H89 treated cells

under capacitating conditions, thus, this needs to be fully explored in other to determine whether this pathway can induce or supports progressive motility of PKA inhibited cells.

The work presented in this thesis also shows that identified compounds have no detrimental effects on human sperm functions and physiology. Although to a large extent this suggests that the compounds are "sperm-safe", the clinical safety and effectiveness of these compounds on human oocytes during human IVF and different stages of embryo development needs to be well established before they can be considered for clinical use. Also, while data from the animal IVF model used in this study suggest that Ibudilast might enhance penetration rate during porcine IVF the effectiveness of Ibudilast over caffeine and other related compounds currently in vogue needs to be tested using the experimental protocol used in this study so as to determine if Ibudilast is a better alternative to compounds which are currently in use.

Finally, the HPLC technique described in this study serves as a proof of concept that this method can be used for the determination of low level of cyclic nucleotides found in mammalian sperm. The use of this technique needs to be expanded upon (and extended to human sperm analysis), that is, for the determination of other nucleotides. This high throughput technique could help improve our knowledge of the intracellular dynamics of cyclic nucleotides in mammalian sperm. An added advantage of the HPLC technique is that it can be coupled to a mass spectrometer, thereby aiding further analysis of unidentified intracellular compounds yet to be identified due to limitation of current methods (such ELISA and radioimmunoassay).

Chapter 8.

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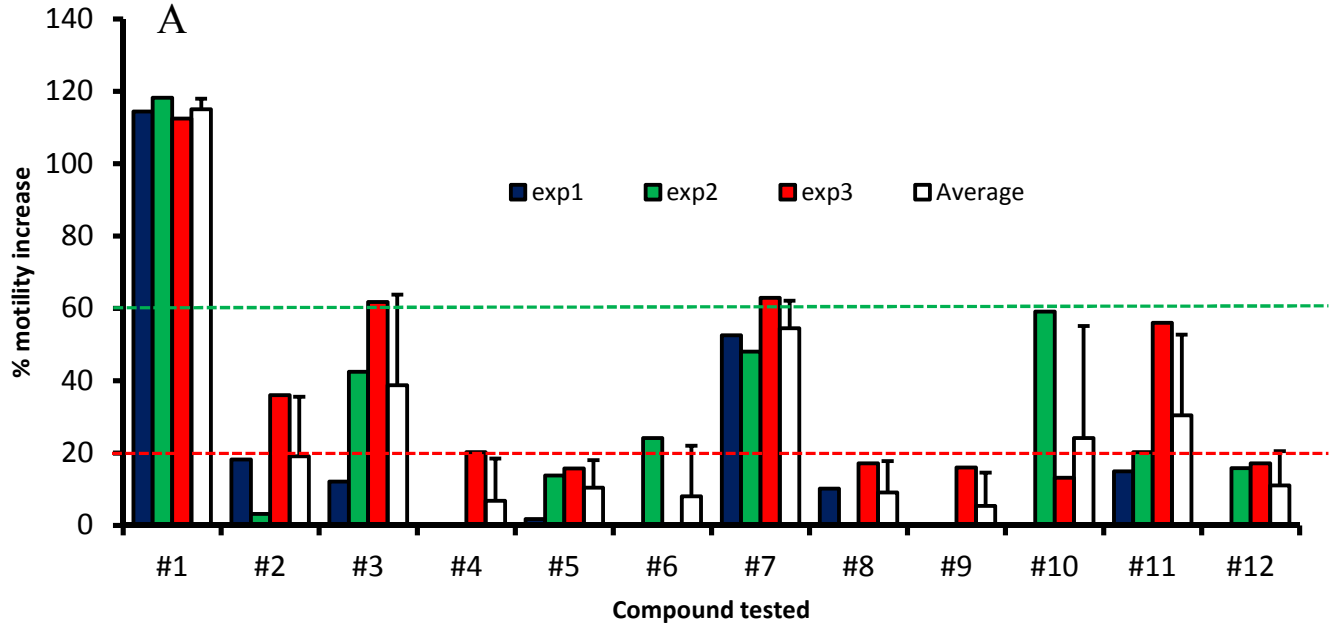
APPENDIX 1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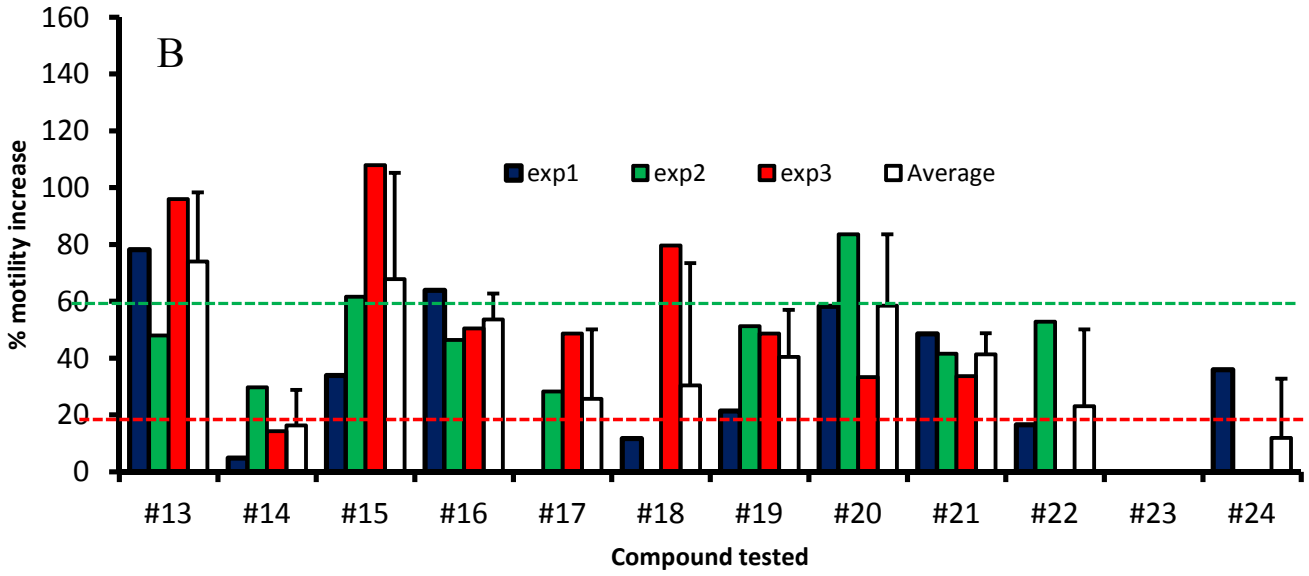
Compound tested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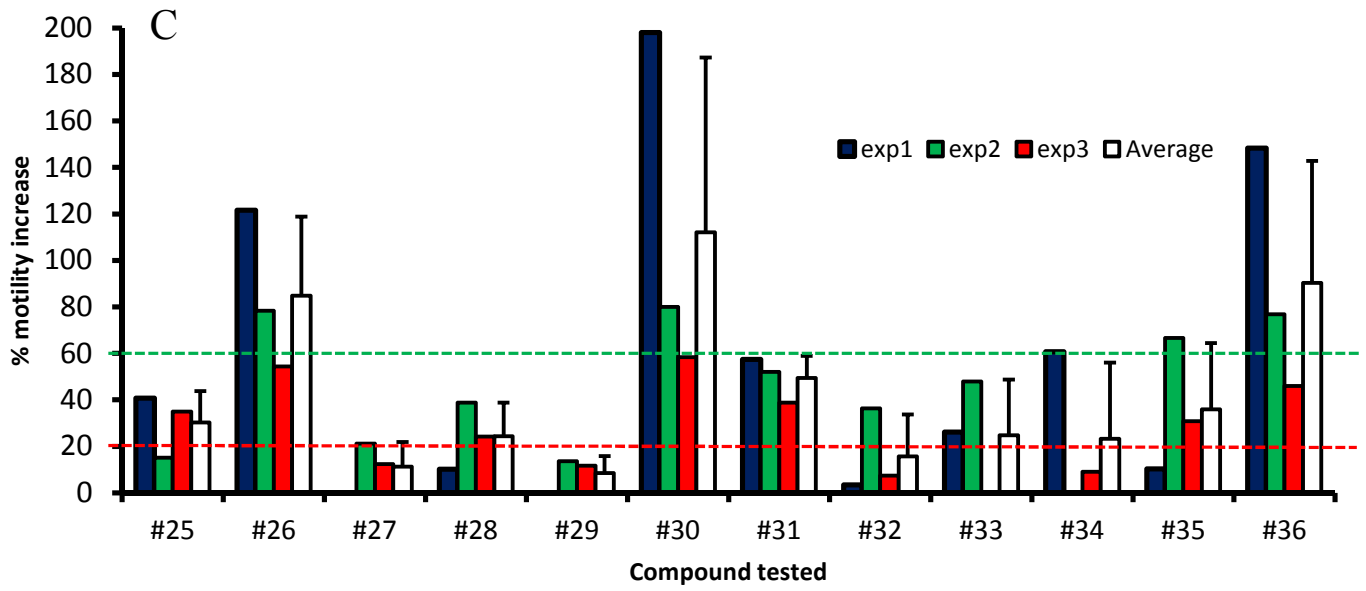
Compound tested



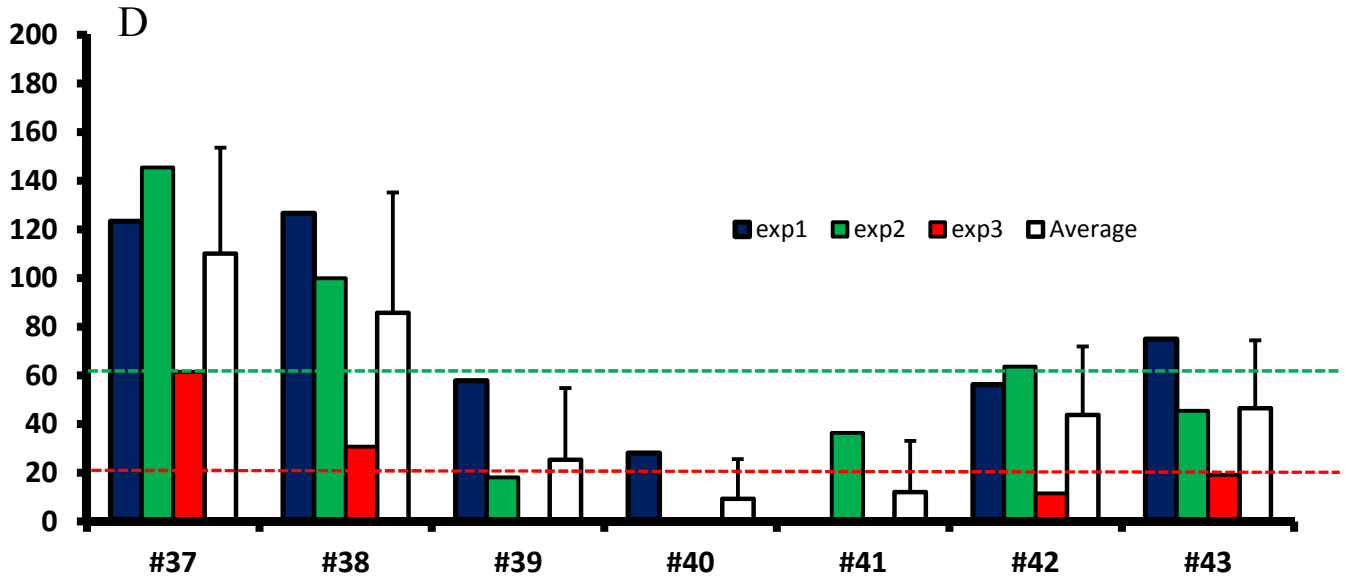


Fig 1: Effect(s) of compound(s) on sperm motility: Human spermatozoa were treated for 20 min at 37°C with 100µM of Compound. (A) Compound tested (#b, #1 to #12). (B) Compound tested (#13 to #24). (C) Compound tested (#25 to #36). (D) Compound tested (#37 to #43). 100% increase equal 2 fold increase in motility compare to 1% DMSO (i.e.: DMSO=15%-- treatment 30%). Green line indicates threshold for strong responder while red line is the threshold for background. Compound between green and red line indicates mild responder. n=3 (3 separate analysis on pooled sample) mean ±SD.

APPENDIX 2

Calculation of limit of quantification (LOQ) and limit of detection (LOD)

	Peak Area	Concentration g/10mL
Buffer A	11813979	0.001
	11813979	0.001
	11813979	0.001
	1186667	0.0001
	1186667	0.0001
	1186667	0.0001
	171994	0.00001
	171994	0.00001
	171994	0.00001
	11512	0.000001
	11512	0.000001
	11512	0.000001
	136	0.0000001
	136	0.0000001
	136	0.0000001

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999990211
R Square	0.999980423

Adjusted R Square	0.999978917
Standard Error	21908.67737
Observations	15

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	3.18722E+14	3.19E+14	664018.7	5.40795E-32
Residual	13	6239871875	4.8E+08		
Total	14	3.18729E+14			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	14983.40652	6507.825867	2.302367	0.038489	924.1034935	29042.70954	924.1034935	29042.71
X Variable 1	11798551487	14478998.5	814.8735	5.41E-32	11767271512	11829831462	11767271512	1.18E+10

LOQ	5.51578E-06
LOD	1.82021E-06

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*
1	11813534.89	443.8033241
2	11813534.89	443.8033241
3	11813534.89	443.8033241
4	1194838.555	-8171.800262
5	1194838.555	-8171.800262
6	1194838.555	-8171.800262

7	132968.9214	39024.9429
8	132968.9214	39024.9429
9	132968.9214	39024.9429
10	26781.958	-15270.03589
11	26781.958	-15270.03589
12	26781.958	-15270.03589
13	16163.26167	-16026.91007
14	16163.26167	-16026.91007
15	16163.26167	-16026.91007

	Peak Area	Concentration g/10mL
Buffer B	11973342	0.001
	12029653	0.001
	12021529	0.001
	1237522	0.0001
	1236312	0.0001
	1232810	0.0001
	121084	0.00001
	121583	0.00001
	121758	0.00001
	12071	0.000001
	12143	0.000001
	12159	0.000001
	0	0.0000001
	0	0.0000001
	0	0.0000001

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999992899
R Square	0.999985797
Adjusted R Square	0.999984705
Standard Error	18983.28259
Observations	15

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	3.29839E+14	3.3E+14	915290.7	6.71561E-33
Residual	13	4684745233	3.6E+08		
Total	14	3.29843E+14			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	8259.588492	5638.856941	1.464763	0.166748	-3922.421302	20441.59829	-3922.421302	20441.6
X Variable 1	12002541293	12545664.7	956.7083	6.72E-33	11975438032	12029644554	11975438032	1.2E+10

LOQ	4.69805E-06
LOD	1.55036E-06

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*

1	12010800.88	-37459.27137
2	12010800.88	18852.45593
3	12010800.88	10728.13186
4	1208513.718	29008.60219
5	1208513.718	27797.83876
6	1208513.718	24295.99809
7	128285.0014	-7201.461238
8	128285.0014	-6702.416879
9	128285.0014	-6527.027069
10	20262.12979	-8191.521636
11	20262.12979	-8118.851758
12	20262.12979	-8102.949022
13	9459.842621	-9459.842621
14	9459.842621	-9459.842621
15	9459.842621	-9459.842621

	Peak Area	Concentration g/10mL
Buffer C	19098941	0.001
	19250200	0.001
	19328191	0.001
	1891354	0.0001
	1912691	0.0001
	1916200	0.0001
	192660	0.00001
	193223	0.00001

194879	0.00001
14274	0.000001
18252	0.000001
18874	0.000001
2307	0.0000001
1986	0.0000001
2158	0.0000001
457	0.00000001
436	0.00000001
446	0.00000001

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.999984205
R Square	0.99996841
Adjusted R Square	0.999966435
Standard Error	41969.04487
Observations	18

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	8.92089E+14	8.92E+14	506465.6	1.94785E-37
Residual	16	28182411641	1.76E+09		
Total	17	8.92117E+14			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*

Intercept	-3010.091425	11085.50224	-0.27153	0.789453	-26510.30637	20490.12352	-26510.30637	20490.12
X Variable 1	19227532462	27017713.79	711.6639	1.95E-37	19170257467	19284807456	19170257467	1.93E+10

LOQ	5.76543E-06
LOD	1.90259E-06

RESIDUAL OUTPUT

Observation	*Predicted Y*	*Residuals*
1	19224522.37	-125581.0124
2	19224522.37	25677.62573
3	19224522.37	103668.3245
4	1919743.155	-28389.01433
5	1919743.155	-7052.06199
6	1919743.155	-3543.060677
7	189265.2332	3394.906913
8	189265.2332	3957.314922
9	189265.2332	5613.701034
10	16217.44104	-1943.439923
11	16217.44104	2034.144936
12	16217.44104	2656.703601
13	-1087.338179	3394.193688
14	-1087.338179	3073.674896
15	-1087.338179	3245.550834
16	-2817.8161	3274.8161
17	-2817.8161	3253.8161
18	-2817.8161	3263.8161

APPENDIX 3

Consent form for patient/donor participation in research

ASSISTED CONCEPTION UNIT

WARD 35

NHS TAYSIDE

NINEWELLS HOSPITAL

DUNDEE DD1 9SY

Direct line (01382) 632111

Fax (01382) 633853

CONSENT FORM FOR PATIENTS/DONORS

[producing extra semen samples]

Title of research: Understanding the regulation of human sperm function and the development of novel treatments for male infertility.

First of all we would like to thank you very much for taking part in our research project.

The aim of this study is to understand how a sperm cell is activated in response to secretions from the female tract - progesterone and nitric oxide and to understand if this activation is abnormal in some men. In addition we would like to test enzyme inhibitors to see if we can enhance sperm motility and hope that in the future we may be able to develop drugs which may be able to improve IVF success.

You may decline to take part, or withdraw at any time without this affecting, in any way, your treatment and care now or in the future.

I have fully understood what will be involved in the project. This study involves me producing a semen (sperm) sample by masturbation in the Assisted Conception Unit or by arrangement at home, for the research purposes of the project. In the future there may be requests for further semen samples.

Signed…….

Name (block capitals)……..

Date……

Witnessed……(name)……Signature

If you have any further queries or questions you can contact either: Mr Steven Mansell (01382 660111 ext. 33605) or Nurse Evelyn Barratt, e.barratt@dundee.ac.uk

APPENDIX 4

Publications:

TARDIF, S., **MADAMIDOLA, O**. A., BROWN, S. G., FRAME, L., LEFIÈVRE, L., WYATT, P. G., BARRATT, C. L. R. & MARTINS DA SILVA, S. J. (2014) Clinically relevant enhancement of human sperm motility using compounds with reported phosphodiesterase inhibitor activity. Human Reproduction.

CAMPBELL, J. M., SAVAGE, A. L., **MADAMIDOLA, O**., TAMHANE, K., SORIANO, R., ADYA, A. K. & BROWN, S. G. (2013) Progesterone significantly enhances the mobility of boar spermatozoa. BioDiscovery, 5

Conference presentation and posters

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